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EDITED BY

PEYTON ROUS
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VINCENT P. DOLE
FRANK L. HORSFALL, JR.



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THE JOURNAL OF EXPERIMENTAL MEDICINE

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SIMON FLEXNER, M.D.

PEYTON ROUS, M.D.

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by way of the biliary tract in all probability (lack of staining of the urine, red coloring of the feces, absence of coloring of the intestinal walls and of the gastric contents, red coloring of the intestinal contents). Similar findings were also obtained in rabbits stained by intravenous injections. The elimination was like that of brilliant vital red (Smith (2)), which from many points of view resembles Congo red and vital red.

3. Slight diffusion through the capillary vessels of the subcutaneous and peri-articular connective tissues and disappearance of this coloring after from 3 to 4 days.

4. No "vital" staining of the reticulo-endothelial system. However, it seems that Congo red in pathological conditions (tuberculosis, Wedekind (8) and Löwenstein (9); sepsis, Adler and Reimann (10)) is a vital dye. It is an acid dye like most other vital stains, and of the benzidinic series.

5. A long permanence *in situ*, in the case of subcutaneous injections of the dye.

EXPERIMENTAL

Material Used.—(a) Merck's Congo red, 1 per cent in H_2O .

(b) Solution of mono-urate of sodium prepared according to Rondoni (11) containing about 1 mg. of mono-urate for each cubic centimeter (titration with $KMnO_3$ N/20) at pH 7.4, as determined colorimetrically by Michaelis' method and electrometrically.

(c) Solution of uric acid with 1 per cent of glucose and Li_2CO_3 0.28 per cent, according to Koehler (12), containing 1 cg. of uric acid for each cubic centimeter, at pH 8–8.2.

(d) Controls.—Buffer solutions of phosphates according to Sørensen at corresponding pH; solutions of electrolytes at various pH; solutions of glucose and Li_2CO_3 , as in the solution of Koehler; inert granular materials.

Method of Injection.—The most clear cut results were obtained by injecting at the same time both the uric acid and the dye (1 cc.) into the peritoneal cavity, killing the animals after 18–24–36 hours. Repetition of the dose was undesirable because of the inflammatory action on the peritoneum of uric acid and the consequent fixation of the dye (Menkin (7)). Studies of the speed of absorption of the dye from the subcutaneous tissue in the presence of uric acid yielded complicated results because of local inflammatory manifestations.

Results.—With intraperitoneal injections of Congo red at the same time as uric acid, an intense staining was found when the animals

THE INFLUENCE OF URIC ACID ON THE PERMEABILITY OF MEMBRANES

BY VIRGILIO CHINI, M.D.

(From the Laboratory of General Pathology of The Royal University of Milan,
Milan, Italy)

PLATE I

(Received for publication, August 1, 1930)

During investigations concerning the action of uric acid upon the tissues, and especially upon vital staining of them with trypan blue (1), the question arose of whether uric acid has an influence on the permeability of cellular membranes. The relatively pronounced staining noticed in animals treated with uric acid and with trypan blue did not provide the answer to this question, since the uric acid brought about alterations of the organs eliminating the dye (kidneys). It seemed probable that the obstacle to elimination resulted in a relatively great concentration of the dye in the blood and an unusual partition of the dye between the plasma and the tissues, according to an equilibrium proved by Smith (2). Dyes have since been used which are only slightly eliminated through the kidneys or not at all, for example, Congo red or vital red (Dawson, Evans and Whipple (3)). Treatment with uric acid can give rise to alterations also of the liver when it is given in large doses or for a long time; and the liver is the probable organ of elimination for Congo red (personal researches). We have tried to avoid the complicating factor by the use of small doses only.

For the study of the biological action of uric acid, rats were chosen as especially suitable on account of their peculiar purin metabolism (4). The following phenomena were noted.

1. Rapid and complete absorption of the dye from the peritoneal cavity within 24 hours.

2. Passage into the circulation, probably through the lymphatic tracts (rapid staining of the mediastinal glands,—see researches of Muscatello (5), Opie (6) and Menkin (7)); and subsequent elimination

It has seemed advisable to carry out some control researches *in vitro*.

Diffusion of Dyes through Gelatin as Affected by Uric Acid

Schulemann (20) noted that the speed with which some dyes are diffused in gelatin is proportional with the speed of their passage through the capillary walls, a fact afterwards confirmed also by

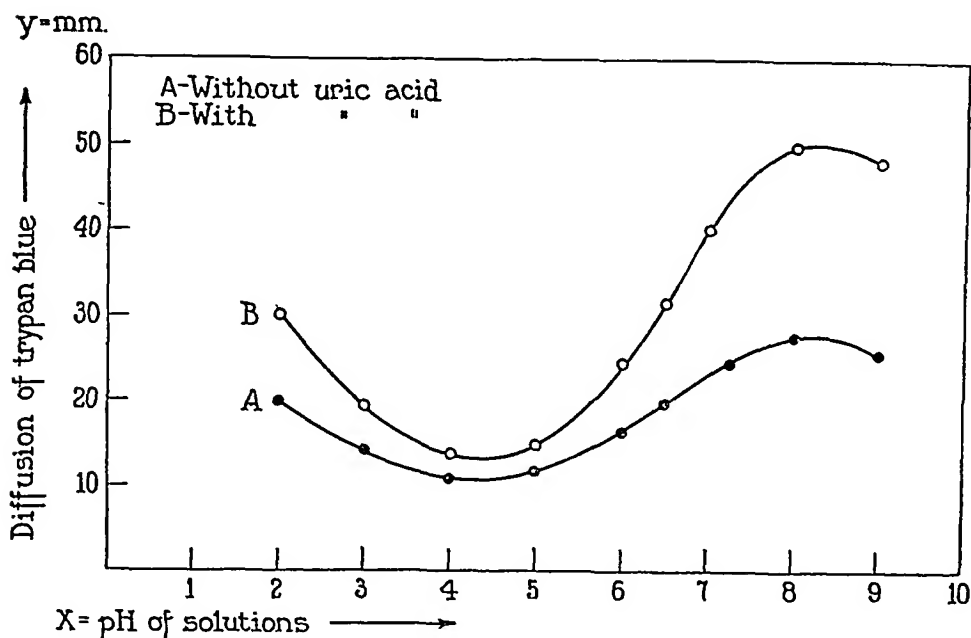


CHART 1. Diffusion of trypan blue in 5 per cent gelatin at different pH of buffer solutions, with or without the presence of uric acid (Koehler's solution). y = millimeters of column of diffusion of the dye. x = pH of buffer solutions.

Krogh (21). We must thank Höber (22) and his pupil Gellhorn (23) for systematic studies on this subject.

I have used bacteriological gelatin which had been dialyzed for 24 hours (Bechhold (24)), brought to various concentrations in H_2O , filtered when hot and distributed in equal amount in graduated bacteriological test tubes. The level attained was marked and represented the zero line. A 0.5 cc. of 1 per cent trypan blue or Congo red in H_2O was poured on the solidified gelatin, with or without uric acid (in solution or suspended). Numerous controls were prepared. The following is a scheme of the experiment.

were killed after 24 hours. It was diffuse in the subcutaneous tissue and especially marked in the peri-articular connective tissue. In control animals, *i.e.*, in animals injected with the control solutions instead of uric acid, only a slight rosy shade of the connective tissues could be obtained.

In the case of subcutaneous injections in corresponding situations, of Congo red plus uric acid, and of Congo red plus control material, we found a greater extension of the surface and depth of the red blotch where uric acid had been introduced, and this blotch disappeared sooner than that on the control side. It is uncertain whether this is due to a larger surface of absorption or to a higher permeability.

DISCUSSION

The interpretation of these results is not simple. Has the uric acid by itself modified the permeability? Or has it brought into play other substances influencing the capillaries? The skin is rich in histamine (Thorpe (13)) and in that histamine-like substance, if it is not true histamine, which goes under the name of the H substance of Lewis (Kalk (14)). Storm van Leeuwen (15) has shown that uric acid can exercise an activating or sensitizing action on various allergic substances, which often behave like shock-poisons. An increased concentration of the circulating dye due to modifications of its elimination must also be thought of. I did not notice any histological modifications of the liver or of the kidneys, but there may have been some functional modifications of these organs. The fact must not be overlooked that the subcutaneous and the connective peri-articular tissues showed a relatively great coloring. Are the capillary vessels of the connective tissues the most permeable? This tissue is largely influenced by modifications of the ion-concentration of the medium (Rous (16)) and may act as a "compensation-compartment" for H-ions present in abnormal quantities in the organism (Chini (17)). Recent researches of Melli (18) have shown that electrolytes introduced direct into circulation are quickly fixed in the subcutaneous tissues. Perhaps in my experiments the uric acid was in a higher concentration in the connective tissues than elsewhere. However, Bergami (19) has shown that a rapid and diffuse fixation of uric acid takes place in the kidneys of rabbits. The behavior of the peri-articular tissues is of particular interest.

The difference in the grade of diffusion, between the tubes of the gelatin, with or without uric acid solutions, is quite evident, even when the controls are varied by adjusting the pH, and introducing various electrolytes or mixtures of NaOH plus citrate of sodium, according to Sørensen.

TABLE I

Diffusion of Trypan Blue 1 Per Cent in H₂O, at Different Gelatin Concentrations

	No. of test tubes							
	I	II	III	IV	V	VI	VII	VIII
Gelatin at 10 per cent, cc.....	20	18	16	14	12	10	8	6
H ₂ O, cc.....	—	2	4	6	8	10	12	14
Concentration per cent of the gelatin.	10	9	8	7	6	5	4	3
Diffusion of trypan blue after 4 days, in mm.....	4	5	6	8	10	12	17	22

TABLE II

Diffusion of Colloidal Dyes Together with Buffer Solutions with or without the Presence of Uric Acid (See Also Chart I)

	5 per cent gelatin in test tubes					
	I	II	III	IV	V	VI
pH of buffer solutions.....	3.8	4.8	5.8	6.8	7.8	8.8
Diffusion of trypan blue, mm.....	15	13	15	25	28	25
Diffusion of trypan blue with uric acid (Koehler's solution).....	18	15	20	45	52	50
Diffusion of Congo red.....		7	9	14	17	13
Diffusion of Congo red with uric acid (Koehler's solution).....		10	16	17	19	20

Dialysis Experiments

Dialysis experiments carried out through an animal membrane (fish's air bladder) have clearly shown the influence of uric acid on the diffusion of trypan blue.

Five dialysis bags, which had proved impermeable to trypan blue beforehand, were placed in a like number of flasks, each containing the same quantity of H₂O. The conditions and the results are given in the following table (Table III).

To a series of test tubes containing 5 per cent gelatin are added 0.5 cc. of trypan blue or Congo red solution plus 2 cc. of $M/3$ phosphate mixture according to Sørensen at various pH (pH 4.8-5.8-6.8-7.2-8.8) plus 1 cc. of Rondoni's solution or that of Koehler. Instead of this last the controls received 1 cc. of H_2O or of physiological salt solution. Other controls were prepared with solutions of various electrolytes at various pH in order to avoid the influence of the phosphate ions on the precipitation of the uric acid (Rondoni (11)); and with granular inert materials.

The column of diffusion of the dye in the gelatin in a given time and at a constant temperature was measured from the zero line in millimeters. Also the grade of diffusion of the dye in gelatin at various dilutions was noted. Bechhold (24) advises breaking up the gelatin into various segments, liquefying it and then making comparative colorimetric readings.

Results.—In conformity with the *in vivo* observations the uric acid was found to favor a greater diffusion of the dyes in the gelatin, not only under conditions of acidity but also under those of alkalinity. This diffusion proved to be a function of the concentration of uric acid.

Certain facts complicated the results even in the controls:

1. The speed of diffusion of the dyes varied inversely as the concentration of the gelatin; graphically, a curve is obtained of a linear relation.

2. When the concentration, time and temperature are constant, the speed of diffusion is a function of the pH of the solution. We may represent this behavior graphically by a partially parabolical curve with a minimum around pH 4-5, and with a greater development towards high values of pH (Chart 1). As the minimum of diffusion corresponds with the values of pH near those of the isoelectric point of the gelatin, it is probable that the diffusion has a relation to the state of imbibition of the colloid, which is naturally at a minimum at the isoelectric point. For the gelatin, the latter corresponds to $[H] = 2.5 \times 10^{-5}$ (Schade), then $[H] = -5 \times 10^{-0.39} = 10^{-5+0.39}$, therefore pH = 4.61.

3. In the tubes of gelatin in which alkaline mixtures of phosphates or the uric acid solution of Koehler have been stratified, phenomena of periodic precipitation can be noticed in the middle of the gelatin, which probably have an influence on the diffusion of the dye.

Some records of observations follow (Tables I and II).

The colorimetric reading could not be made with a standard sample of trypan blue, because the dialyzed fluid had taken a violet shade during the first days, which does not appear in saline or H₂O solutions of trypan blue, even when diluted to the extent of 1:1,000,000. I have noticed a certain metachromasia of the trypan blue also in the first grades of diffusion of the color in the gelatin (pomegranate red color), which was very marked in alkaline vehicles and absent in acid vehicles. After 2 or 3 days, the concentration of the trypan blue in the liquid dialyzed when uric acid is present, is such as to take the characteristic blue color, and it can therefore be compared with the standard samples of trypan blue (comparison is possible with concentrations of more than 1:100,000 (Chart 2)).

In collateral research, it has been established that the pH of the mixture (phosphates) subjected to dialysis as well as granular materials by themselves have no influence on the diffusion of trypan blue.

CONCLUSIONS AND SUMMARY

1. Congo red injected *in vivo* together with uric acid gives rise to more intense and diffuse red coloring in rats, especially in the subcutaneous and peri-articular tissues, than is the case in control rats injected simply with dye.

2. Uric acid added *in vitro* to solutions of Congo red or trypan blue increases the speed of diffusion of these dyes, both through gelatin and the animal membranes (dialyzers).

These results support a view long maintained by Professor Rondoni (25), namely, that some factor of an endothelial-capillary nature must be taken into consideration in manifestations of hyperuricemia and of gout.

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TABLE III

Diffusion through Animal Membranes of Trypan Blue Solutions with Various Admixtures

No. of dialysis	Experiment	Coloring of dialyzed fluids			Colorimetrical readings (mm.)			Observations
		After 24 hours	After 48 hours	After 96 hours	After 24 hours	After 48 hours	After 96 hours	
1	0.5 cc. of trypan blue solution + 2 cc. of Kochler's solution	++	++++	+++++	8	6	2½	
2	0.5 cc. of trypan blue solution + 2 cc. physiological solution	—	—	—	—	—	—	No diffusion
3	0.5 cc. of trypan blue solution + 2 cc. 1 per cent glucose	—	—	—	—	—	—	No diffusion
4	0.5 cc. of trypan blue solution + 2 cc. (1 per cent glucose + 0.28 per cent Li_2CO_3)	+-	+-	+-	40	40	40	
5	0.5 cc. of trypan blue solution + 2 cc. 1 per cent uric acid suspension in H_2O	+-	++	++++	50	8	2	

Colorimetrical readings with Duboscq colorimeter modified by Leitz.

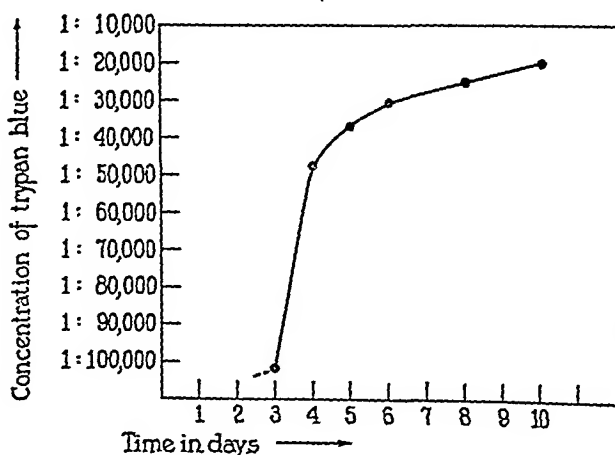


CHART 2. Concentration of trypan blue in the dialyzed liquid compared to standard solutions between concentrations 1:100,000-1:10,000. In the dialysator 0.5 cc. of 1 per cent trypan blue solution had been suspended with 2 cc. of Kochler's solution.



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EXPLANATION OF PLATE 1

FIG. 1. *On the left:* Rat injected in the peritoneal cavity with 1 cc. of 1 per cent Congo red solution + 1 cc. of physiological solution. *On the right:* Rat of same weight injected also in the peritoneal cavity with 1 cc. of 1 per cent Congo red solution + 1 cc. of uric acid solution of Koehler. Rats killed after 18 hours from injection. Stronger staining of subcutaneous tissues in the rat having received uric acid in addition to dye.

FIG. 2. Diffusion in the 4 per cent gelatin of trypan blue solution with buffer solutions of phosphates at different pH:

1 and 1' = pH 7.8

2 and 2' = pH 8.8

1' and 2' with addition of uric acid (Koehler's solution).

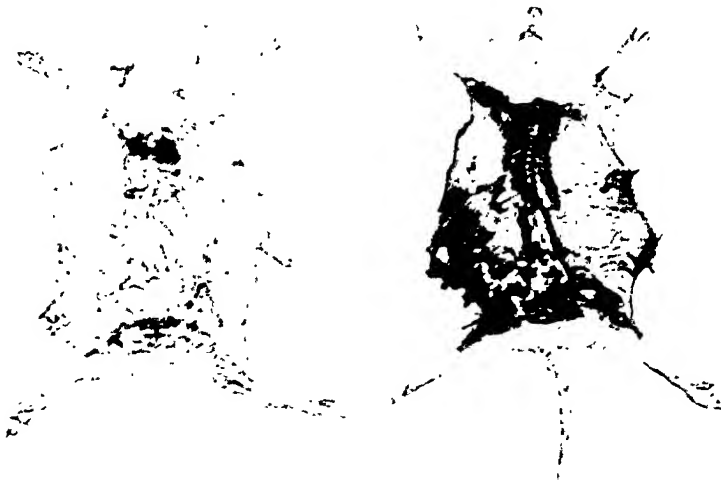


FIG. 1



FIG. 2

(Chini: Uric acid and permeability of membranes)

Diet 20 consisting of mash, made by a local dealer, and supplemented with skimmed milk powder, cod liver oil and yeast.

Stock Diet 20

		<i>per cent</i>	<i>per cent</i>
Mash	Coarse yellow corn meal.	27.5	81.0
	Wheat bran.	20.0	
	Wheat flour middlings.	15.0	
	Ground oats.	13.5	
	Alfalfa leaf meal.	7.5	
	Bone meal.	5.0	
	Skimmed milk powder.	3.5	
	Calcium carbonate.	2.0	
	Sodium chloride.	1.0	
	"Meato".	5.0	
	Skimmed milk powder.	15.0	
	Cod liver oil.	2.0	
	Yeast, Fleischmann's bakers' dried.	2.0	

The similarity of these three diets with respect to their calculated nutritive value is shown in Table I.

Diets 107 and 108 are seen to differ only in the ratio of fat to carbohydrate. The protein in the diet is of animal origin, save that supplied by the yeast. As regards vitamin content, all diets include sufficient cod liver oil to satisfy the requirements for vitamin A and D. Vitamin B complex is furnished by 5 per cent Fleischmann's dried yeast and milk powder in the simplified diet, and by the cereals and milk powder in the stock diet. No provision is made for vitamin C (Hart, Halpin and Steenbock (2)); and while Diets 107 and 108 are undoubtedly low in vitamin E, the stock diet contains ample. On June 18, when the chicks were 10 days old, the stock diet was replaced in increasing proportions by Diet 107 or Diet 108. On June 29, the stock diet was entirely withdrawn, and the chicks given only Diet 107 or 108. Twenty-seven other chicks of the same hatch were continued upon the stock diet, and served as controls. Up to the present time, they have shown excellent growth and normal appearance and behavior. The peculiar disorder which forms the subject of this paper, was observed only in chicks maintained partly or wholly during their growing period upon the simplified diets.

Reference to the accompanying growth chart will show that the general growth of the chicks on Diets 107 and 108, while not so good

A CEREBELLAR DISORDER IN CHICKS, APPARENTLY OF NUTRITIONAL ORIGIN*

BY ALWIN M. PAPPENHEIMER, M.D., AND MARIANNE GOETTSCH, Ph.D.

WITH THE ASSISTANCE OF ANNE PAPPENHEIMER

(From the Departments of Pathology and Biochemistry, Columbia University, New York, and the Storrs Agricultural Experiment Station, Storrs, Connecticut)

PLATES 2 TO 4

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In the course of certain experiments having for their object a study of vitamin E deficiency in fowls, an interesting nervous disorder appeared amongst two small groups of White Leghorn chicks. The disease was manifested only in birds receiving certain simplified diets; it was accompanied by characteristic symptoms and by uniform and well defined lesions of the cerebellum.

I. Diets.—The simplified diets were similar to those used by Evans and Burr (1) in vitamin E studies upon the rat, except that roughage was supplied by the inclusion of 10 per cent of paper pulp (Hart, Halpin and Steenbock (2)). Their composition was as follows:

<i>Group I. Diet 107</i>		<i>Group II. Diet 108</i>	
	<i>per cent</i>		<i>per cent</i>
Skimmed milk powder, Merrel			
Soule.....	15	15
Casein, Merck's technical....	15	20.5
Cornstarch.....	45	20
Lard.....	3	21
Cod liver oil, Mead's.....	2	2
Yeast, Fleischmann's bakers'			
dried.....	5	5
Salt mixture, McCollum 185 (3).	5	6.5
Paper pulp, Eastman.....	10	10

The chicks were hatched on June 8, 1930. Before being placed upon these simplified diets, they were started in batteries upon the stock

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stationary, or declined, as one might expect from the inability of the birds to secure food.

II. Clinical Behavior.—Symptoms pointing to a derangement of function in the central nervous system appeared in many of the chicks with great suddenness after they had been on the diet 3 or 4 weeks. They suddenly became prostrated, lying with legs outstretched and spastic, claws flexed, and head retracted and often twisted, sometimes through a complete semi-circle (Fig. 1). Clonic spasms of the legs were often observed; sometimes coarse tremors. The eyelids drooped, and in the late stages, there was somnolence or stupor. Some of the chicks showed rotary movements to right or left, and before they became completely prostrated, many of them became incoordinate and ataxic, making rocking movements on their legs on rising, or tilting the body to one side or other. It is worth noting that even in the terminal stages of the disease, a true paralysis of the wings or legs never occurred.

The course, duration and intensity of the symptoms varied greatly in different individuals. Death occurred in one chick (7623) on the same day that the symptoms first appeared, and several others were killed on the first day when apparently moribund; others survived for varying intervals up to 16 days. In these, there were transient periods of improvement. As is shown in Table II a few birds showed very trivial incoordination which disappeared as they grew older.

The time elapsing from the institution of the diet to the first appearance of symptoms ranged from 9 to 38 days, the greatest number coming down with symptoms after they had been from 18 to 25 days on the diet. The youngest chick was 19 days old, the oldest 48. In one chick (7641) the symptoms first appeared while it was still receiving small amounts of the stock diet.

III. Pathological Changes.—The lesions found at autopsy were in general limited to the cerebellum, although in two instances, observed in a later experiment, they occurred only in the cerebrum. In most of the birds the alterations were readily detected with the naked eye, the cerebellum appearing swollen, softened, the convolutions flattened or malformed, and discolored reddish or brownish in the affected areas (Fig. 2).

as the controls, compares favorably with that given as "normal" by Card and Kirkpatrick (4), and exceeds that given as "nearly normal"

TABLE I
Nutritive Value of Diets

Diet	Per cent indigestible	Per cent protein	Per cent COH	Per cent fat	Per cent ash	No. of calories per 100 gm.	Nutritive ratio	Ca/P ratio
107	10.0	18.2	54.6	5.2	6.7	338	1:3.6	1.5
108	10.0	22.8	26.6	23.2	8.4	407	1:3.4	1.5
Stock 20	13.5	14.9	42.6	5.1	10.6	285	1:3.8	1.8

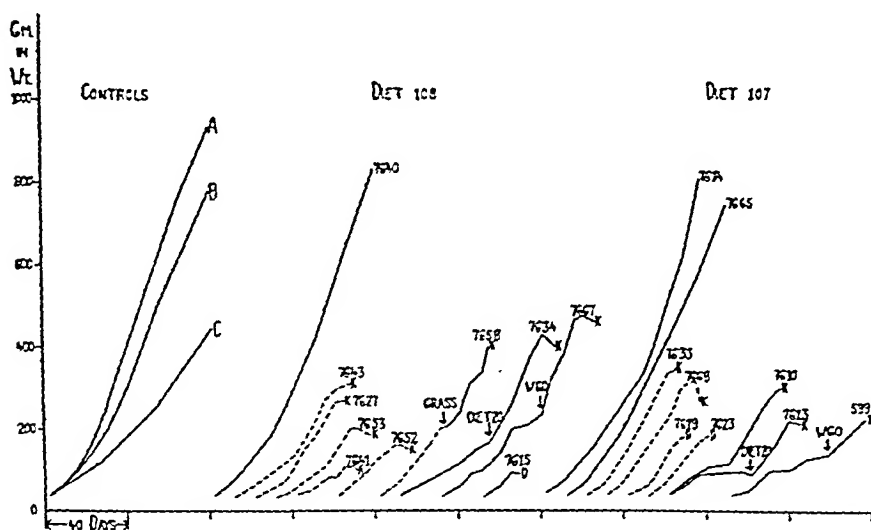


CHART 1. Showing rate of growth of chicks on Diets 107 and 108. Chicks showing cerebellar disorder are charted in dotted line.

A—Composite growth curve of 27 control chicks on Diet 20.

B—Composite curve given by Card and Kirkpatrick (4) as "normal" for chicks.

C—Composite curve given by Hart, Halpin and Steenbock (2) as "nearly normal" for chicks.

by Hart, Halpin and Steenbock (2). With the appearance of the characteristic symptoms, the weight curve in most cases remained

definitely shrunken and pycnotic. In places, they are undergoing fragmentation with the formation of small globular chromatin particles. The fibers of the central white matter are disrupted by edema.

In addition to these purely regressive changes, there is a mild inflammatory reaction. In the meninges, and about many of the small vessels, there is an increased number of mobile mononuclear cells, both lymphoid elements and large macrophages with vacuolated cytoplasm. One of the latter is found in mitosis.

No bacteria are seen, and no definite cell inclusions can be recognized.

The *medulla* contains a small recent area of hemorrhage situated in the floor of the 4th ventricle near the lateral recess. There are no other pathologic changes. Sections through cerebrum, optic lobes, spinal cord and dorsal ganglia show nothing abnormal. The other viscera, and skeletal muscles show nothing abnormal.

Chick 7653.—On July 18, suddenly became prostrate and helpless. Lay with legs outstretched, head twisted (Fig. 1). When disturbed, made forced circular movements to left. Killed by decapitation.

Autopsy: On removing brain, a small hemorrhage was noted in cerebellum, the rest of the brain appearing grossly normal. There were small blotchy hemorrhages into the leg muscles, and into serosa over duodenum. The viscera were otherwise normal in appearance.

Microscopic: Cerebellum. The most striking feature is a zone of edema and rarefaction between the granular and molecular layers. The cells in this region are widely separated and the tissue is spongy—in places completely disrupted. Purkinje cells show marked degeneration. Many are densely stained with pycnotic shrunken nuclei and condensation of the Nissl substance. Others show vacuolar or hydropic swelling of the nucleus. Still others are completely necrotic, all nuclear structure being lost, and the entire cell reduced to an amorphous purplish or eosin staining mass. There are small hemorrhages, but a mononuclear reaction, such as was present in Chick 7652, is not found. Hyaline thrombi are seen within the capillaries in the more severely degenerated areas.

Sections through remainder of central nervous system—cerebrum, optic lobes, basal ganglia, medulla, spinal cord and dorsal root ganglia were carefully studied, but no lesions were found in H-E preparations. There were no visceral lesions.

Chick 7627.—Definite symptoms were first noted on June 18, when it was found helpless and stuporous, with head retracted. Unable to stand, but there were spastic movements of the legs, and no paralysis. The chick was killed by decapitation the same day, and the cerebellum removed. A part was used for the inoculation of other chicks, the remaining fragment fixed in 95 per cent alcohol. Grossly no lesions were noted at the time, but the fragment when sectioned after hardening, showed definite softening and brownish discoloration over a large area. Under the dissecting microscope, the tissue presented worm-eaten or a eroded surface in contrast to the surrounding smooth normal tissue.

Microscopically, sections through the fragment showed large areas of edema,

The microscopic lesions, though varying greatly in their extent from small focal areas to large confluent patches involving the greater portion of the cerebellum (Fig. 3), were nevertheless strikingly uniform in character. The essential changes, which will be illustrated in greater detail by the presentation of individual protocols, were: (1) edema, with separation and disruption of the cellular and fibrillar elements; (2) degeneration and necrosis of the Purkinje cells, and of the small cells constituting the granular layer; (3) small hemorrhages scattered through the central white matter, or within the cortical zones; (4) hyaline capillary thrombi in and about the necrotic areas (Figs. 4, 5, 6).

The lesions in the early cases were thus typically degenerative or regressive in character. An inflammatory reaction was noted in but one case, a chick which survived for 12 days after the onset of the symptoms. In this instance, the cellular reaction was exclusively mononuclear, and of slight intensity.

Interesting reparative changes were observed in Chick 7658, which recovered after being given grass, and in which the previously degenerated tissue showed gliosis and calcification.

All other regions of the brain and cord appeared to be free from obvious lesions, although it is probable that the application of more refined neurological technique may disclose minor changes secondary to the cerebellar injury. With the routine methods thus far employed, they have not been noted.

The following protocols represent typical examples of the disease.

Chick 7652.—On June 28, was found reeling and badly picked by other birds in cage. Isolated. July 1, improved, and returned to cage. July 12, again incoordinate and weak. July 14, dying; spastic contractions of legs, with claws flexed. Head retracted. Stuporous. Crop empty. Killed.

Autopsy: No significant gross changes were found in the abdominal or thoracic viscera. The brain showed *softening and brownish discoloration of the cerebellum.*

Microscopic: The brain was fixed in 95 per cent alcohol. Sections through cerebellum disclosed marked lesions. There were numerous hemorrhages, chiefly in the white matter. Many of the Purkinje cells are degenerated, or actually necrotic, having lost their nuclear staining and being reduced to formless eosin staining masses. Hyaline thrombi are found in the capillaries in the affected areas, although the alcohol fixation makes one cautious as to this. The nuclei of the granular layer are more widely spaced than in the normal areas, and are

The demarcation of the necrotic areas from the adjacent healthy tissue is in some places abrupt, in others gradual, but there is no limiting reaction. Fatty granule cells are not observed. The meninges are not noticeably altered, although there are one or two small extravasations of red cells, and about one of the cerebellar arterial branches, there is a heavy collar of lymphoid cells.

The medulla and optic lobes, which are included in the section, show excellent fixation and complete absence of lesions. Sections through cerebrum, corpus striatum, brachial enlargement of spinal cord show no lesions. The visceral sections are without interest.

These five cases, strikingly similar in their clinical behavior and in the character of the cerebellar lesions discovered at autopsy, are illustrative of those birds which die or are killed during the active period of the disease. But recovery may take place after a transient exhibition of symptoms, and the following protocol shows well the nature of the residual lesions in such a recovered case.

Chick 7658.—On July 3, 15 days after beginning the administration of Diet 107, the bird showed incoordination and weakness, and was badly picked by other birds in cage. It was isolated and given fresh greens. On July 18, it was still ataxic, swaying and stumbling when it walked. It continued to gain in weight, and remained in fair condition until August 1, when it was killed for examination.

Autopsy: One of the cerebellar lobules was sunken below the level of the adjacent lobules, and had an intense brownish color. The adjacent lobules had a pitted surface, and were also slightly brownish. Upon transection, after fixation in 10 per cent formalin, this brownish area was seen to extend 3 to 4 mm. into the substance of the cerebellum. Nothing otherwise abnormal was found.

Microscopic: An entire cerebellar lobule is found to have shrunk and degenerated, the normal zones and elements being replaced by an indifferent spongy glial tissue in which the cellular elements have no orderly distribution. In the better preserved areas, scattered Purkinje cells are recognizable, but many of them are impregnated with calcium. Many small irregular masses of calcium are scattered through the degenerated area. There are also numerous spongy phagocytes containing clumps of nonrefractile greenish brown pigment, which does not resemble hemosiderin (Fig. 7).

The atrophy and shrinkage of the cerebellar lobules is associated with marked edema of the overlying pia arachnoid.

Other blocks taken through the anterior portion show large areas of gliosis with pigmentation, loss of zonal architecture, disappearance of Purkinje cells. The region of the nucleus lateralis is severely affected.

Amongst the birds which we have studied, there have been two in which the lesions were localized in the cerebrum, and not in the

rarefaction and necrosis, without hemorrhage or inflammatory reaction. The remainder of the brain and the thoracic and abdominal viscera were normal.

Chick 7653.—Apparently well until June 22 when it was found lying on its side, with legs extended and spastic, head retracted and eyes closed. Stuporous, but struggled when aroused. Killed.

Autopsy: On exposing the brain, the caudal portion of the cerebellum was covered by edematous pia, and had a striking bluish discoloration, evidently from recent hemorrhage. This extended to the lateral aspects. The remainder of the brain, spinal cord and sciatic nerves were grossly normal. The thoracic and abdominal viscera were also normal.

Microscopic: Cerebellum. Whereas a portion of the cerebellar cortex represents a normal structure, the greater part has undergone extensive softening, and can be distinguished from the healthy tissue in the stained section by its lighter color. The lesions are identical with those seen in previous cases. Edema and disruption of fibers, degeneration or complete necrosis of Purkinje cells, pyknosis of the nuclei of the cells of the granular layer, scattered hemorrhages, and very extensive capillary thrombosis throughout the softened areas. The thrombi are particularly striking in sections stained with Mallory's phosphotungstic acid hematoxylin. There are no perivascular cell accumulations or other evidence of inflammatory reaction (Fig. 3).

In sections taken through the caudal portion of the cerebellum, the degenerative lesions are less pronounced, but there is massive hemorrhage into the central white matter of the lobules.

The remainder of the brain, spinal cord, sciatic nerve and other viscera exhibit nothing of interest. In the skeletal muscle of the leg, there are areas of edema and acute inflammatory lesions of mild intensity, probably due to the trauma inflicted by other birds in cage.

Chick 7668.—On June 20, found prostrate, legs extended, head twisted and retracted. Forced circular movements. Killed on June 22.

Autopsy: On exposure of brain, a faint yellowish discoloration and swelling of the cerebellum was noted, the rest of the brain and cord appearing normal. Upon transection after hardening in alcohol, a very distinct area of encephalomalacia characterized by softening and brownish color, was found to occupy the greater portion of the cerebellum. The best preserved tissue was on the surface, but in places the softening extended to the meninges. There were no gross visceral lesions.

Microscopically, the cerebellum showed massive necrosis, in places proceeding to actual liquefaction. This involved the entire central white matter, but in many places also the granular and molecular layers, reaching the surface of the lobules. The fibers were disrupted, the nuclei of the cells of the granular layer were shrunken, densely stained, often fragmented into small spherules diminishing in size to that of a large coccus. The Purkinje cells have lost their nuclei, and stain diffusely with eosin. The small capillaries are filled with pink staining hyaline masses.

The study of this bird shows that slight transitory symptoms may occur with recovery, and without permanent anatomical lesions.

As regards the pathogenesis of the cerebellar lesions, we are inclined to regard the capillary thrombosis as the primary cause of the degenerative changes. When India ink was injected into the carotid arteries, the smallest and earliest lesions remained uninjected, so that occlusion of the capillary stream bed in the affected areas appeared to be a constant accompaniment of the lesions, and in all probability, their primary cause.

A general oversight of our material is given in Table II. Of the 19 chicks on Diets 107 and 108 (7614, 7640, 7665), 3 are still alive, having shown no clear cut symptoms of nervous disorder. They have gained normally and aside from lack of pigment in legs and iris, look and behave like healthy birds. Six chicks (7652, 7653, 7658, 7633, 7668) have shown severe and typical symptoms, and have had characteristic changes in the cerebellum. One of these, (7658) however, recovered partially from the symptoms coincidentally with the administration of green grass, and the cerebellar lesions at the time of death, though extensive, were completely healed. One chick (7643) had slight transitory symptoms, and minimal lesions, discovered only on serial section of the entire cerebellum. Four chicks showed characteristic symptoms, but the cerebellum was not studied. Of the five completely negative cases, 2 (7634, 7613) were transferred to the stock diet 20 on July 18, until they were killed after 25 and 32 days respectively; 2 (7667, 599) received wheat germ oil (550 mg. once weekly) by pipette during the period from July 18 until they were sacrificed on the 25th and 30th days. One (7610) received the unmodified Diet 107 throughout.

The fact that the symptoms and lesions developed only in chicks receiving Diet 107 or 108, and that the 27 other chicks of the same hatch maintained on the stock diet showed normal growth and behavior, seemed to incriminate the diet as in some way related to the disorder. However, it seemed desirable to ascertain whether we were dealing with an infectious disease, and the following experiment was planned to test its transmissibility.

On July 18, twelve 11-day old chicks were inoculated subdurally with a thin suspension in saline of the finely divided cerebellum of

cerebellum. One chick was found to have a small area of necrosis in the cerebrum, in addition to the characteristic cerebellar lesions. The following protocol illustrates the cerebral localization of the lesions:

Chick 589.—Inoculated subdurally on July 18 with suspension of cerebellum from Chick 7653. Placed on Diet 108. Has remained stunted. On August 11, it was noted that there was marked tremor of legs, a tendency to turn to the right and to keep right eye closed. There was, however, no marked ataxia, and the symptoms seemed atypical. Killed August 12.

Autopsy: Brain showed opaque whitish areas over convexity of cerebral hemispheres on both sides. The cerebellum appeared externally normal.

Microscopic: There are marked lesions throughout both hemispheres of the cerebrum. These consist in large patches of necrosis, in which nuclear staining is lost. The capillaries are distended with hyaline plugs. Some of the areas are superficial, others are in the substance of the hemispheres, some abut upon the third and lateral ventricles (Fig. 8).

Most of the lesions show more or less replacement of the necrotic tissue at the margins by a loose, spongy and in places very cellular neuroglial tissue, in the meshes of which is much purple granular detritus, probably derived from disintegrated nuclear material. Often this is enclosed within mononuclear phagocytes (Fig. 9). The larger vessels are free from thrombi. The fresh necrotic areas are sharply demarcated from the adjacent healthy tissue, and bordered by fresh hemorrhage. *Cerebellum.* There are no lesions of the usual character found on Diet 108. Some of the Purkinje cells are sclerotic and stain densely, but none show complete necrosis. Optic lobes and medulla likewise normal.

Finally, we may present the case of a chick which remained practically free from symptoms, and in which the anatomical findings in the cerebellum were trifling and equivocal.

Chick 7643.—Received Diet 107 from June 18 to August 4. On July 30, it was noted that the chicken was a bit unsteady on its legs. Nothing else abnormal could be observed. The weight increased normally, and when killed on August 4, the bird seemed in excellent condition.

Autopsy: No gross lesions of brain or viscera.

Microscopic: The cerebellum with medulla and optic lobes attached was cut in complete series of 6 micra, every 10th section being mounted. No lesions were found in any portion of the cerebellum, except in Sections 47 and 48. Here there was an area of rarefaction in the central white matter of the convolutions. The nuclei of the glia cells in this area were slightly shrunken and denser than elsewhere. There were no hemorrhages, thrombi or necroses.

injection—convulsive movements, retraction of the head, followed by stupor lasting for an hour or more. Two of the inoculated chicks died a few minutes after the injection, and one after several hours. The three survivors recovered slowly. A control chick inoculated

TABLE III

Showing Incidence of Cerebellar Disease in Inoculated Chicks on Simplified and Natural Food Diets

Chick No.	Diet	Source of injected material	Days on diet until		Clinical symptoms	Lesions	
			Appearance of symptoms	Death		Gross	Microscopic
574	20	Chick 7653	—	D 6	—	—	—
575	20	" 7653	—	K 46	—	—	—
581	20	" 7653	—	K 27	—	—	—
582	20	" 7653	—	D 10	—	—	—
595	20	" 7653	—	D 6	—	—	—
598	20	" 7653	—	K 39	—	—	—
971	20	" 7627	—	K 23	—	—	—
959	20	" 7627	—	K 23	—	—	—
583	108	" 7653	26	K 26	++	+++	+++
584	108	" 7653	13	K 13	++	+	++
588	108	" 7653	15	K 15	++	++	++
							(Cerebrum also)
590	108	" 7653	21	K 21	++	+	++
592	108	" 7653	18	K 19	+	—	+
589	108	" 7653	24	K 25	+	++	++
							(Cerebrum only)
987	108	" 7627		D 3	—	—	—
992	108	" 7627		D 3 hrs.		Not examined	
972	108	Saline	23	K 23	++	+++	+++

with 0.1 cc. of sterile saline showed no symptoms following the injection.*

* One of the writers has had occasion in the course of work on Fowl Paralysis, to inoculate large numbers of chicks subdurally with suspensions of brain and spinal cord. A severe immediate reaction to the injection of 0.1 cc. of suspension has never been observed. It would seem that the cerebellar suspension used in this experiment was highly toxic—possibly because of the products of autolysis present in the necrotic and softened tissue.

Chick 7653—a case showing typical acute symptoms and lesions. All survived the injection, although three showed transient shock. On July 22, 5 additional chicks of the same hatch, now 15 days old,

TABLE II
Showing Incidence of Cerebellar Disease in Chicks on Simplified Diets

Chick No.	Diet	Days on diet until		Clinical symptoms	Lesions	
		Appearance of symptoms	Death		Gross	Microscopic
7634	108 ⁽¹⁾	—	K 62	—	—	—
7640	108	35	Alive	±	—	—
7641	108	9	K 21	+++	Brain not examined	—
7643	108	17	K 17	±	—	±
7652	108	12	K 27	+++	++	Serial sections
7653	108	30	K 30	+++	+	+++
7658	108 ⁽²⁾	15	K 43	++	++	++
7667	108 ⁽³⁾	—	K 58	Recovery	—	+++
7615	108	—	D 10	—	—	Healed lesion
7627	108	34	K 34	+++	Brain not examined	—
7633	107	38	K 38	+++	—	—
7665	107	—	Alive	+++	++	++
7668	107	32	K 34	+++	++	+++
599	107 ⁽³⁾	—	K 55	+	—	+++
7613	107 ⁽¹⁾	—	K 54	Atypical	—	—
7614	107	—	Alive	—	—	—
7619	107	18	D 18	+++	—	—
7623	107	20	D 20	+++	Brain not examined	—
7610	107	—	K 49	—	Brain not examined	—

⁽¹⁾ Diet 20 substituted on July 23.

⁽²⁾ Grass was given on July 10 and 11.

⁽³⁾ 550 mg. of wheat germ oil given on July 23, 30, and Aug. 7.

were injected subdurally with 0.1 cc. of a saline suspension of the cerebellum of Chick 7627, the tissue being ground with sand in a sterile mortar. All showed severe immediate symptoms following the

Three additional chicks of the same hatch were placed upon Diet 108 at the age of 23 days. One (1845) was found dead after 18 days with extensive softening of the cerebellum; a second (1820) showed slight ataxia and tremors on the 23rd day and was killed. In the cerebellum were found multiple discrete early lesions. The third chick (1892) showed no symptoms but was also sacrificed on the 23rd day. No lesions were found in the brain.

DISCUSSION

That the development of this cerebellar disease is related in some way to the diet is sufficiently obvious. Only chicks which received Diets 108 and 107 acquired the disease; controls of the same hatch kept in intimate contact under identical environmental conditions, but receiving only natural food diets, remained free. The nature of the relationship is, however, by no means clear. Among the possibilities which have presented themselves, and which may be briefly considered at this time, are the following:

1. That the disease is due to an infective agent, to which only the chickens on the simplified diet are susceptible. This assumption seems far fetched. The failure to produce the disease by direct subdural inoculation of cerebellar suspension, unless the animals are kept on the simplified diet, makes it impossible to prove or disprove this theory, but against it is the purely *regressive* or *degenerative* character of the pathological picture, as well as the absence of microorganisms in the lesions, or of cell-inclusions suggestive of a virus infection.

2. That the disease is the result of the absorption of some toxic substance from the intestinal tract. The diet itself may contain some substance which is poisonous for the growing chick; or the bacterial flora may be modified by the character of the diet to the detriment of the animal.

3. That the disease is a true nutritional deficiency disease. The satisfactory growth of the birds would indicate that the caloric, protein, and mineral requirements had been adequately covered. (See Table I.) As regards the known vitamins, a deficiency of A and D can be definitely excluded. Vitamin C has been shown by Hart, Halpin and Steenbock (2) to be unnecessary for the chick.

Of the 16 chicks which survived the inoculation, 8 were placed upon the stock Diet 20, and 8 upon Diet 108. The results are summarized in Table III, from which it will be seen that amongst the former group, none of the birds displayed symptoms, or showed pathological changes upon gross and microscopic examination. In striking contrast to this, is the result amongst the group receiving Diet 108. Excluding one chick which died from unknown cause on the third day after injection, every animal of this group manifested

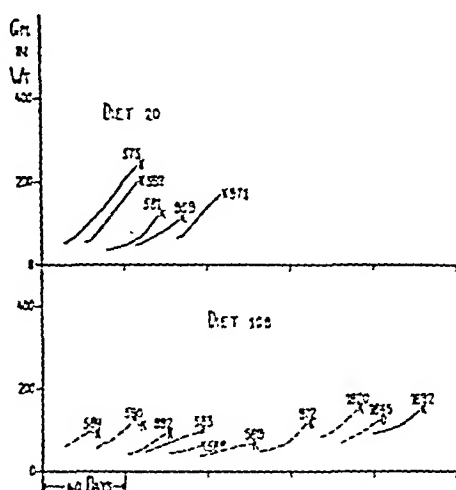


CHART 2. Showing rate of growth of chicks on Diets 20 and 108 which had been inoculated with brain from affected birds. Also 3 uninjected chicks of the same hatch.

more or less characteristic nervous symptoms, appearing from the 13th to the 26th day, and all showed definite and pronounced lesions of the brain.

The growth of the chicks in this experiment (Chart 2) was not satisfactory. They were obtained from a different source and many of them, including controls, were infected with coccidia.

One may deduce from this experiment that the disease is not transmissible by subdural inoculation to chicks maintained on the stock diet.

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EXPLANATION OF PLATES

PLATE 2

FIG. 1. Positions assumed by affected chicks. Note retraction and twisting of head.

FIG. 2. Chick 583. Transection through cerebellum, showing extensive area of softening. (Approximately $\times 7$ diam.)

FIG. 3. Chick 7633. Low power showing extensive softening and rarefaction in central portion of cerebellum.

PLATE 3

FIG. 4. Chick 1820. Section through margin of small degenerating area. To the left, normal nuclei of granular layer; to the right, pycnotic nuclei more widely spaced because of edema. A capillary containing hyaline thrombus and two degenerating Purkinje cells are seen at junction of molecular and granular layers.

FIG. 5. Chick 7652. Section through degenerating area in cerebellum, showing necrosis of ganglion cells, pycnosis and fragmentation of granular layer and hemorrhage.

FIG. 6. Chick 1820. Thrombosis of capillary, beginning rarefaction and degeneration of granular layer. (High power.)

PLATE 4

FIG. 7. Chick 7658. Extensive atrophy of entire lobe of cerebellum, with gliosis, calcification, and edema of overlying pia arachnoid. (Low power.)

FIG. 8. Chick 589. Extensive area of recent necrosis in cerebrum.

FIG. 9. Chick 589. Replacement of necrotic area in cerebrum by spongy glial tissue.

The diet is low in vitamin E, and indeed was devised for the purpose of studying this deficiency in the fowl. As regards the need for the vitamin B complex, B, G, and "B₂" of Williams (5), this may or may not have been satisfied by the incorporation of 5 per cent of Fleischmann's bakers' dried yeast in the diet. The character of the lesions, however, would seem to differentiate the disease from the polyneuritis due to B deficiency.

In this connection, it is interesting to note that Hogan and Shrewsbury (6), using a similar type of simplified diet, observed spasms and tremors of the legs and head retraction, and even "cartwheel" movements which they interpret as "typical neuritic symptoms." However, even with the inclusion of 40 per cent of dried yeast in their diet, 3 out of 7 chicks in one experiment developed symptoms within 3 weeks. It seems improbable that they were dealing with a vitamin B complex deficiency. Since the central nervous system was not studied one can only surmise that they were concerned with the same disease as that here described.

A condition simulating polyneuritis in chicks has been attributed by Hughes, Lienhardt and Aubel (7) to lack of vitamin A, but their histological studies have not yet been published. In our experiments, a deficiency of vitamin A can be excluded.

CONCLUSIONS

Growing chicks maintained on a diet consisting of milk powder, casein, starch, yeast, cod liver oil, salts and filter paper develop ataxia, tremors, retraction or twisting of the head, clonic spasms of the legs, and stupor. These symptoms may appear suddenly, usually between the 18th and 25th day, and may end in death. If recovery takes place, the chicks may go on to normal development.

Definite lesions are found in the cerebellum of the affected chicks. These consist of edema, necrosis and hemorrhages. Hyaline thrombi are found in the capillaries in and about the degenerated areas.

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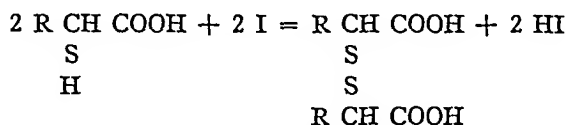




Preparation of the Mercapto and Disulfo Soaps

Three methods of preparing the α -mercapto fatty acids were tried: the method of Eckert and Halla (1913), which consists of refluxing the α -brom soaps with alcoholic NaSH; the method of Nicolet and Bate (1927) in which the brom fatty acids are converted to the pseudothiohydantoin by thiourea and then broken down by boiling with alkali; and the method of Lovén and Johansson (1916), in which the brom soaps are treated with potassium xanthogenate and the resulting compound broken down by ammonia. The best yields were obtained by the method of Lovén and Johansson. The mercapto acids were purified by crystallization from petroleum ether; the acid with eight carbon atoms required the use of solid CO₂.

The disulfo acids are easily prepared from the mercapto acids by oxidizing the latter with iodine in alcoholic solution, as follows:



The calculated amount of iodine is added to the mercapto acid in alcohol. The proper quantity is checked by testing with starch-potassium iodide. The disulfo acids are also crystallized from petroleum ether.

The melting points of these acids were found to be as follows:

	Mercapto acids	Disulfo acids
Caprylic.....	oil	oil
Capric.....	47°C.	37°C.
Lauric.....	59°C.	48°C.
Myristic.....	66°C.	56°C.
Palmitic.....	72°C.	63°C.
Stearic.....	80°C.	72°C.

The mercapto and disulfo acids are much stronger acids than the parent unsubstituted acids. Their soaps are therefore very little hydrolyzed by water; both sodium and potassium soaps, even of the long chained acids, are very soluble.

The technic of doing the germicidal tests has been fully described in previous papers and need not be repeated here.

DISCUSSION

The number of carbon atoms in the molecule of these soaps markedly influences germicidal action (Tables 1 and 2, and Figs. 1 and 2). The germicidal titers increase rapidly with the length of the chain until a maximum is reached, then the titers fall off. The most active soap in the series is not always the same for the different test organ-

THE GERMICIDAL ACTION OF α -MERCAPTO AND α -DISULFO SOAPS

By ARNOLD H. EGGERTH,* PH.D.

(From the Department of Bacteriology, Hoagland Laboratory, Brooklyn)

(Received for publication, September 15, 1930)

The soaps are in many respects the most interesting of all germicides. Some of them, such as the oleate and the bromopalmitate, are among the most powerful and rapid germicides known. It is possible that certain of the soaps play an important part in the body defence against bacteria (see Flexner's introduction to Lamar, 1911). In at least one disease—leprosy—a soap has been found to have a specific chemotherapeutic value. The chief interest of the soaps, however, lies in the fact that they demonstrate to an unusual degree the phenomenon of selective germicidal action; they are, therefore, ideally suited to a study of the effect of chemical structure on such selective action. The selective action of the oleates has been known for some time (Avery, 1918). In previous studies reported by the writer (Eggerth 1926, 1929 a and b) numerous instances have been given in which one soap was highly toxic for one species of bacteria and of low toxicity for a second species, while another soap acted in just the reverse manner on the same two species. One interesting experiment may be recalled here: a mixture was made of four species of bacteria; then by employing four different soaps, any one of the four species could be killed at will without destroying the remaining three (Eggerth, 1929 b). Such a high selective action is unique among germicides.

In the hope of extending our knowledge of this interesting phenomenon, the writer has prepared and tested two series of sulfur-containing soaps: the α -mercapto and the α -disulfo soaps, which are the subject of this paper.

* Van Cott Fellow, Department of Pathology, Hoagland Laboratory.

TABLE 1—*Concluded*

	α -Mercapto- caprylate	α -Mercapto- caprate	α -Mercapto- laurate	α -Mercapto- myristate	α -Mercapto- palmitate	α -Mercapto- stearate
No. of carbon atoms.....	8	10	12	14	16	18
<i>Vibrio cholerae</i>						
pH						
6.5	N/320	N/1280	N/10,240	N/5120	N/320	0
7.5	N/80	N/320	N/2560	N/2560	N/320	N/80
8.5	N/20	N/160	N/1280	N/1280	N/640	N/160
<i>B. typhosus</i>						
6.5	N/40	N/40	N/40	0	0	0
7.5	N/20	N/40	N/80	N/80	0	0
8.5	N/10	N/40	N/80	N/160	0	0
<i>B. pyocyaneus</i>						
6.5	0	0	N/40	0	0	0
7.5	0	0	N/40	0	0	0
8.5	0	0	N/40	0	0	0

Time of tests, 2 hours; temperature, 37°C.

isms. Thus, at pH 7.5, in the α -mercapto series (Table 1), the mercaptolaurate is the most active soap against *Micr. ovalis* and *B. pyocyaneus*; the mercaptolaurate and mercaptomyristate are equally active against *B. diphtheriae*, *B. lepi-septicus*, *B. melitensis*, *Vibrio cholerae*, and *B. typhosus*; while the mercaptomyristate is the most active against *Diplococcus pneumoniae*, *Strep. haemolyticus*, and *Staph. aureus*. In the disulfo series of soaps (Table 2), the dicaprate is most germicidal for *B. lepi-septicus*, *Vibrio cholerae*, and *B. typhosus*; the dilaurate is most germicidal for *B. diphtheriae*, *Staph. aureus*, *Micr. ovalis*, and *B. melitensis*; and the dimyristate is most germicidal for *Diplococcus pneumoniae* and *Strep. haemolyticus*.

With other series of soaps a similar close relationship between germicidal action and number of carbon atoms in the molecule has been shown. Thus, in the unsubstituted normal aliphatic series, the soaps with 12 and 14 carbon atoms are most germicidal; in the α -brom series, those of 12, 14, 16, and 18 carbon atoms are most active; in the α -hydroxy series, it is those with 14 and 16 carbon atoms (Eggerth

TABLE 1
The Germicidal Titers of α -Mercapto Soaps

	α -Mercapto- caprylate	α -Mercapto- caprate	α -Mercapto- laurate	α -Mercapto- myristate	α -Mercapto- palmitate	α -Mercapto- stearate
No. of carbon atoms.....	8	10	12	14	16	18
<i>Diplococcus pneumoniae</i>						
ϕH						
6.5	n/320	n/5120	n/81,920	n/327,680	n/10,240	n/2560
7.5	n/160	n/2560	n/20,480	n/81,920	n/10,240	n/5120
8.5	n/80	n/1280	n/10,240	n/40,960	n/20,480	n/5120
<i>Streptococcus haemolyticus</i>						
6.5	n/320	n/2560	n/20,480	n/5120	n/1280	n/640
7.5	n/80	n/1280	n/10,240	n/20,480	n/1280	n/640
8.5	n/80	n/640	n/10,240	n/10,240	n/2560	n/1280
<i>B. diphtheriae</i>						
6.5	n/320	n/1280	n/10,240	n/10,240	n/1280	n/640
7.5	n/80	n/640	n/10,240	n/10,240	n/1280	n/640
8.5	n/40	n/640	n/5120	n/10,240	n/2560	n/1280
<i>Staphylococcus aureus</i>						
6.5	n/40	n/320	n/1280	n/2560	n/80	0
7.5	n/20	n/80	n/640	n/1280	n/40	0
8.5	0	n/20	n/640	n/1280	n/20	0
<i>Micrococcus ovalis</i>						
6.5	n/40	n/320	n/1280	n/640	0	0
7.5	n/20	n/80	n/1280	n/640	n/40	0
8.5	n/10	n/80	n/1280	n/640	n/160	0
<i>B. leptisepticus</i>						
6.5	n/320	n/2560	n/5120	n/5120	0	0
7.5	n/80	n/640	n/2560	n/2560	n/40	0
8.5	n/80	n/640	n/1280	n/2560	n/160	n/80
<i>B. melitensis</i>						
6.5	n/80	n/640	n/10,240	n/10,240	n/160	0
7.5	n/80	n/320	n/5120	n/5120	n/160	0
8.5	n/20	n/80	n/2560	n/5120	n/320	0

TABLE 2—*Concluded*

	α -Disulfodi- caprylate	α -Disulfodi- caprate	α -Disulfodi- laurate	α -Disulfodi- myristate	α -Disulfodi- palmitate	α -Disulfodi- stearate
No. of carbon atoms.....	2 x 8	2 x 10	2 x 12	2 x 14	2 x 16	2 x 18
<i>Vibrio cholerae</i>						
pH						
6.5	N/320	N/1280	N/160	0	0	0
7.5	N/40	N/160	N/40	0	0	0
8.5	N/40	N/80	N/20	0	0	0
<i>B. typhosus</i>						
6.5	0	0	0	0	0	0
7.5	0	N/20	0	0	0	0
8.5	0	0	0	0	0	0
<i>B. pyocyaneus</i>						
6.5	0	0	0	0	0	0
7.5	0	0	0	0	0	0
8.5	0	0	0	0	0	0

Time of tests, 2 hours; temperature, 37°C.

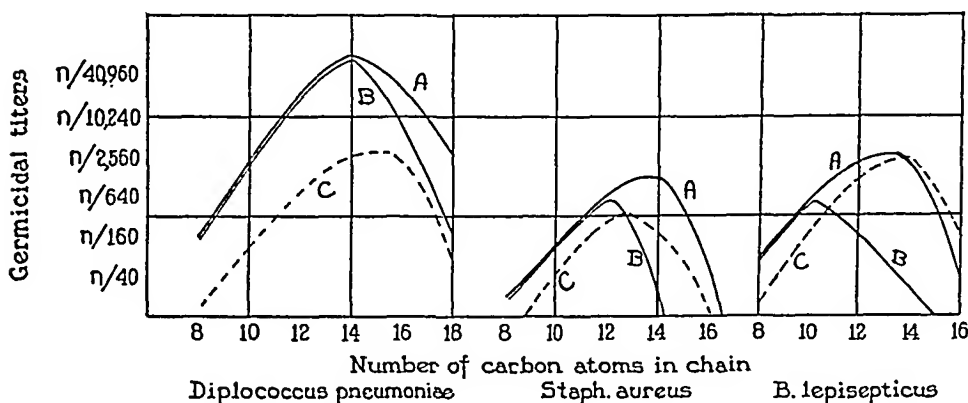


FIG. 1. The germicidal titers of α -mercapto soaps (A), α -disulfo soaps (B), and of unsubstituted saturated soaps (C). The soaps are designated by the number of carbon atoms in their molecule. Time of test, 2 hours; temperature, 37°C.; the pH is 7.5.

TABLE 2
The Germicidal Titers of Disulfo Soaps

No. of carbon atoms.....	α -Disulfodi-caprylate	α -Disulfodi-caprate	α -Disulfodi-laurate	α -Disulfodi-myristate	α -Disulfodi-palmitate	α -Disulfodi-stearate
	2 x 8	2 x 10	2 x 12	2 x 14	2 x 16	2 x 18
<i>Diplococcus pneumoniae</i>						
pH						
6.5	N/320	N/2560	N/40,960	N/81,920	N/10,240	N/320
7.5	N/160	N/1280	N/20,480	N/81,920	N/5120	N/160
8.5	N/80	N/1280	N/10,240	N/81,920	N/5120	N/160
<i>Streptococcus haemolyticus</i>						
6.5	N/320	N/2560	N/20,480	N/20,480	N/160	0
7.5	N/80	N/1280	N/10,240	N/20,480	N/640	0
8.5	N/80	N/640	N/10,240	N/10,240	N/640	0
<i>B. diphtheriae</i>						
6.5	N/320	N/1280	N/20,480	N/640	0	0
7.5	N/80	N/640	N/10,240	N/1280	N/320	0
8.5	N/40	N/320	N/10,240	N/5120	N/640	0
<i>Staphylococcus aureus</i>						
6.5	N/40	N/160	N/1280	0	0	0
7.5	N/20	N/80	N/640	0	0	0
8.5	N/10	N/40	N/640	0	0	0
<i>Micrococcus oralis</i>						
6.5	N/40	N/320	N/1280	0	0	0
7.5	N/20	N/160	N/1280	N/80	0	0
8.5	N/10	N/160	N/640	N/320	0	0
<i>B. lepioteticus</i>						
6.5	N/320	N/2560	N/320	0	0	0
7.5	N/80	N/640	N/160	N/40	0	0
8.5	N/80	N/640	N/160	N/80	0	0
<i>B. melitensis</i>						
6.5	N/80	N/640	N/2560	N/640	0	0
7.5	N/20	N/320	N/2560	N/320	0	0
8.5	N/20	N/80	N/640	N/320	0	0

melitensis, and *B. typhosus*; with the other test organisms in this series, it is less germicidal than the brom soap. Fig. 2 shows that the α -mercaptomyristate is a very powerful germicide, comparing favorably with any other soap tested by the writer. It is curious that the mercaptopalmitate and -stearate should be such feeble germicides; these soaps are readily soluble in water and titers comparable with the bromopalmitates and -stearates were expected.

The titers of the disulfo soaps bear an interesting relationship to those of the mercapto soaps. On comparing Table 2 with Table 1, it will be observed that with the lower members of this series, the

TABLE 3

The Germicidal Titers of Potassium Myristate, Potassium α -Bromomyristate, Potassium α -Mercaptomyristate, and Potassium α -Disulfodimyristate

Test organism	Myristate	α -Bromomyristate	α -Mercaptomyristate	α -Disulfodimyristate
<i>Diplococcus pneumoniae</i>	N/2560	N/40,960	N/81,920	N/81,920
<i>Streptococcus haemolyticus</i>	N/640	N/10,240	N/20,480	N/20,480
<i>B. diptheriae</i>	N/320	N/5120	N/10,240	N/1280
<i>Staphylococcus aureus</i>	N/160	N/5120	N/1280	0
<i>Micrococcus ovalis</i>	N/40	N/2560	N/640	N/80
<i>Vibrio cholerae</i>	N/160	N/1280	N/2560	0
<i>B. melitensis</i>	N/320	N/2560	N/5120	N/320
<i>B. leptisepticus</i>	N/2560	N/5120	N/2560	N/40
<i>B. typhosus</i>	0	N/20	N/80	0
<i>B. pyocyaneus</i>	N/40	N/20	0	0

All tests at pH 7.5; time, 2 hours; temperature, 37°C.

titers are usually identical with those of the corresponding mercapto soaps; the higher members are consistently weaker germicides. In no case are the titers of the disulfo soaps higher than the corresponding mercapto soaps. This suggests that, because of their higher molecular weight, the disulfo soaps penetrate less readily; and that those that do enter the bacterial cell are promptly reduced to the mercapto form.

Examination of Fig. 2 brings out what is one of the most interesting facts among the soaps; their highly selective action. In this figure, only the most active and characteristic members of each soap series are given. It will be observed that no two bacterial species run the

1926, 1929 a and b). In an extensive study of numerous cyclo alkyl acetic and dialkyl acetic soaps, Adams and his associates (numerous papers appearing since 1926 in the *Journal of the American Chemical Society*) have shown that maximal germicidal activity toward *B. leprae* occurs in soaps having 16, 17, or 18 carbon atoms.

From the foregoing it seems probable that the following general rule will apply to all soaps: in any homologous series of soaps, germicidal action increases rapidly with the number of carbon atoms in the chain, up to a maximum, after which it diminishes. The point at which this maximum occurs varies with the chemical structure of the soap and with the test organism.

As has been previously suggested by the writer (Eggerth 1929 a) it seems probable that two main factors determine the germicidal action of a soap: its ability to penetrate into the bacterial cell, and its toxic action upon the bacterial protoplasm after it has penetrated. As the number of carbon atoms in the soap molecule increases, the toxicity becomes greater but the power to penetrate into the cell diminishes. As long as molecular size does not prevent entrance of the soap into the cell, germicidal action will increase with molecular weight; eventually a point is reached where the molecule is so large it cannot readily enter the cell, and germicidal action is diminished.

The effect of the pH upon the germicidal action of soaps has been repeatedly noted and discussed by the writer elsewhere (Eggerth 1926, 1929 a and b). Study of the present series does not add anything new to our knowledge on that subject. As the pH often modifies the germicidal titer a great deal, it is obviously necessary that strict attention be paid to it in conducting these tests. As it is impossible to predict in any particular case just what will be the effect of a change in pH upon the soap titer, it is desirable to test out several pH values.

The effect of the introduction of the SH group into the soap molecule is brought out in Table 3 and in Fig. 2. Table 3 shows that the introduction of this group into the myristate radicle markedly increases germicidal action for most of the organisms tested; with *B. lepi-septicus*, the titer is not changed; with *B. pyocyaneus*, it is diminished. Compared with the corresponding brom soap, which it somewhat resembles, it is found to be twice as germicidal for *Diplococcus pneumoniae*, *Strep. haemolyticus*, *B. diphtheriae*, *Vibrio cholerae*, *B.*

clearly distinguished between them. Thus, α -hydroxymyristate is 4 times, and α -disulfodimyristate is 8 times as toxic for *Strep. haemolyticus* as for *B. diphtheriae*; while α -hydroxypalmitate and -stearate, in 18 hours, are 32 times as toxic for *B. diphtheriae* as for *Strep. haemolyticus* (not shown in Fig. 2; see Eggerth, 1929 b).

Such selective reactions should be of use in classifying, isolating, and identifying bacteria. Some work in the direction has been done in this laboratory: cultures of anaerobes and *Sporotrichum* have been freed from contaminating cocci by using bromopalmitate agar; sodium oleate has been used to distinguish between *Micr. ovalis* (called *Strep. fecalis* by many authors) and other streptococci; a probable biological relationship between *Micr. zymogenes* and *Micr. ovalis* has been demonstrated, as they run very similar soap titers. Avery's oleate-haemoglobin agar used in isolating *B. influenzae* is, of course, widely known. Other applications of this selective germicidal action of soaps remain to be worked out.

SUMMARY AND CONCLUSIONS

1. Certain of the α -mercapto soaps and α -disulfo soaps are powerful germicides.
2. In the α -mercapto series, those soaps with 12 and 14 carbon atoms are most germicidal. In the disulfo series, the dicaprates, dilaurates, and dimyristates are most germicidal. The optimum number of carbon atoms varies with the test organism used.
3. These soaps, like others previously studied, show a markedly selective germicidal action.

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same titers with these soaps; conversely, no two soaps give the same titers with these ten test organisms. While certain organisms re-

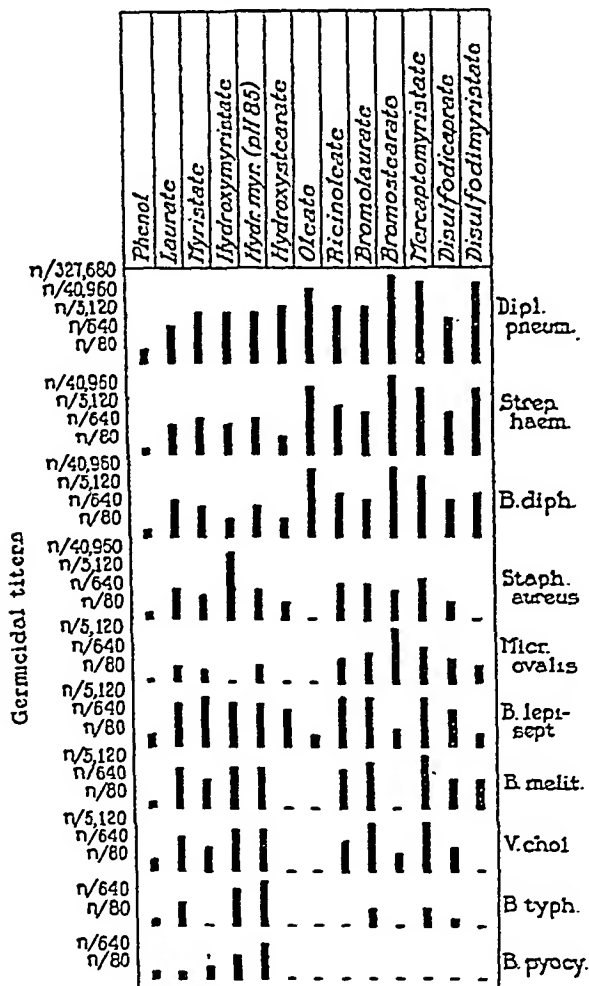


FIG. 2. The germicidal titers of various soaps, showing selective germicidal action. The titers for phenol are included for comparison. The titers of the first nine soaps are taken from previous papers (Eggerth, 1926, 1929 a and b). Time of tests, 2 hours; temperature, 37°C.; the pH is 7.5 except where otherwise indicated.

semble each other in their soap titers (*Strep. haemolyticus* and *B. diphtheriae*; *B. melitensis* and *V. cholerae*), yet certain soaps have always

EXPERIMENTAL

The following tables give the results obtained when bacteria are injected simultaneously with testicle extract into the skin of mice and rats. It should be understood that by + and ++ is meant a lesion of about 1 cm. or even less, indurative, without a genuine abscess formation and disappearing in from 2 to 10 days; by the expression +++ is meant a lesion from 2 to 3 cm. in diameter, distinct oedema,

TABLE I

Organism	Number of mice injected		Average resultant lesion		Number of animals dead	
	Testicle extract	Ringer's	Testicle extract	Ringer's	Testicle extract	Ringer's
<i>E. typhi</i>	2	2	++++	++	2	0
<i>B. dysenteriae</i> Shiga.....	3	3	++++	++	1	0
" " Hiss Y.....	3	3	+++	++	0	0
" " Flexner.....	3	3	++	+	0	0
Cholera.....	2	2	+	—	0	0
<i>B. paratyphosus</i> A.....	2	2	++	—	0	0
" " B.....	2	2	+++	+	0	0
<i>Proteus</i> X19.....	2	2	++++	++++	1	0
<i>Proteus</i> X2.....	2	2	+++	+	0	0
<i>Enterococcus</i>	2	2	+++	+	0	0
<i>Enteritidis</i>	2	2	++++	+	1	0
<i>Tetragenus</i>	2	2	++++	++	2	0
<i>B. prodigiosus</i>	2	2	++++	++	2	0
<i>S. rosea</i>	2	2	+++	++	0	0
<i>Str. erysipelas</i>	3	3	++++	++	1	0
<i>Pneumococci</i> Type III.....	4	4	++++	++	3	0
<i>E. coli</i> , str. 1.....	2	2	+++	+	0	0
<i>E. coli</i> , str. 2.....	2	2	++++	++	1	0
<i>B. violaceus</i>	2	2	++	+	0	0

a central area of suppuration, healing in about 10 to 20 days; and by ++++ is meant a lesion that is acute, with great oedema and with suppuration and ulceration, which may extend the complete length of the abdomen, healing in from 4 to 5 weeks time. These units of degree of infection and the time required for the lesion to heal depend entirely on the virulence of the bacteria used and the dose given.

The effect of the Reynals factor on the lesions produced by many

THE ACTION OF TESTICLE, KIDNEY, AND SPLEEN EXTRACTS ON THE INFECTIVE POWER OF BACTERIA

By M. PIJOAN

(From the Laboratories of Pomona College, Claremont, California)

(Received for publication, June 23, 1930)

Work reported by Duran-Reynals (1) has shown the existence in certain organs of a substance which we shall call, for purposes of abbreviation, the Reynals factor (2), capable of enhancing to an extraordinary degree vaccinal and staphylococcus infections.

The Reynals factor is not species specific and is present in the testicle, the extracts of which, together with the extracts of epididymis, are by far the most active in this respect of any organ. Extracts of kidney, skin, brain and placenta share the property only to a certain extent. Extracts from retina, muscle and ovary do not modify the infection, whereas those of blood, spleen and bone marrow never enhance and sometimes interfere with, and even suppress the activity of the infectious agent.

As the earlier work on the Reynals factor included observations on only two strains of staphylococci (3), it was thought of interest to try many strains and kinds of bacteria and to test the constancy of the factor and whether its action is widespread.

Methods

For the sake of convenience, the method of preparing testicle extract is described again in this paper. Fresh testicle is removed from an anesthetized or freshly killed animal, stripped of its membranes and the glandular tissue is ground in a sterile mortar with an equal quantity by weight of sterile Ringer's solution. It is then centrifuged, and the supernatant fluid is used for the experiments. To this supernatant fluid is mixed varying amounts of a 24 hour broth culture of active bacteria. The type of broth culture and its pH are, within limits, negligible.

Experiments with Kidney Extract.—Extracts of kidney prepared in the same way as those of testicle activate in a lesser degree the staphylococci. So far 24 animals have been inoculated, with 2 strains of staphylococci and kidney extract, including controls. The results from intracutaneous injection into rats of 24 hour broth cultures of

TABLE IV

	Number of rats injected	Dose staph. culture	Kidney extract	Ringer's solution	Lesion	Number animals dead
		cc.	cc.	cc.		
Nonhemolytic	3	0.5	0.5	—	+++	0
	3	1	1	—	+++	0
	3	0.5	—	0.5	+	0
	3	1	—	1	++	0
Hemolytic	3	0.5	0.5	—	+++	0
	3	1	1	—	++++	1
	3	0.5	—	0.5	++	0
	3	1	—	1	+++	0

TABLE V

Resulting lesions from 1 cc. 24 hr. broth culture staphylococci									
Dose spleen extract	Total animals injected	Number of animals with different degree of lesion			Dose Ringer's	Total animals injected	Number of animals with different degree of lesion		
		+	++	+++			+	++	+++
cc.					cc.				
1	5	4	1	0	1	4	1	2	1
1	5	1	4	0	1	4	2	1	1
0.5	7	1	6	0	0.5	5	1	2	2
2	4	3	1	0	2	4	0	2	2

Staphylococcus py. aureus mixed with kidney extract are shown in Table IV.

Experiments with Spleen Extract.—Spleen extract, when added to a suspension of organisms and inoculated intracutaneously into rabbits, has the opposite effect of extracts of testicle and kidney. The lesions are never enhanced, and are in many cases slightly less than those in the controls. The experiments with spleen extract deal only with

kinds of bacteria on mice is clearly shown by the intracutaneous injection of 0.15 cc. of an 18 hour broth (pH 7.8) culture of the following organisms together with 0.15 cc. of testicle extract or Ringer's solution (Table I).

TABLE II

	Number of rats injected	Dose staph. culture	Testicle extract	Ringer's solution	Average resultant lesion	Number animals dead
Nonhemolytic staph.	2	cc.	cc.	cc.		
	2	0.5	0.5	—		
	2	1	1	—	++	0
	2	2	1	—	+++	0
	2	0.5	—	—	+++	0
	2	1	—	0.5	±	0
Hemolytic staph.	2	2	—	1	+	0
				1	+	0
	4	2	2	—	++++	2
	2	3	2	—	++++	1
	4	2	—	2	++	0
	2	3	—	2	++	0

TABLE III

	Rabbit	<i>B. coli</i>	Testicle extract	Ringer's solution	Lesion
Experiment 1.....	1	cc.	cc.	cc.	cm.
".....	"	0.1	1	—	2.5 x 8.4
Experiment 2.....	2	0.1	—	1	1.8 x 4.7
".....	"	0.5	0.5	—	1.8 x 4.7
Experiment 3.....	3	0.5	—	0.5	8.5 x 4.7
".....	"	0.25	0.5	—	4.2 x 2.4
Experiment 4.....	4	0.25	—	—	8.4 x 5.5
".....	"	0.1	0.5	0.5	1.6 x 1.6
".....	"	0.1	—	—	7.7 x 6.1
".....	"	0.1	—	0.5	2.9 x 1.7

The effect of the Reynals factor on lesions produced by a 24 hour broth (pH 7.3) culture of active *Staphylococcus py. aureus nonhaemolyticus* and *haemolyticus* injected together with testicle extract in varying doses intracutaneously into rats is shown in Table II.

The results of intracutaneous inoculation of a 24 hour broth (pH 7.3) culture of active *B. coli* together with testicle extract into rabbits are shown in Table III.

degree than testicle extracts, while spleen extracts never give rise to enhancement, and often cause the lesion to be less than would ordinarily be the case.

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two strains of staphylococci. The extract was prepared in the same way and used in the same manner as the two foregoing extracts.

The results of intracutaneous injection of *Staphylococcus py. aureus* (*haemolyticus*) into rabbits simultaneously with spleen extract are shown in Table V.

DISCUSSION

The observations of Duran-Reynals on the action of different organ extracts on vaccine virus and staphylococcus have been extended in the present experiments to 20 different strains of bacteria. Hoffman (4) has shown that testicle extract enhances the infectivity of herpes virus, vesicular stomatitis and Borna disease. It is apparent, then, that we are dealing with a general phenomenon which may well play a rôle in natural disease.

The nature of the enhancement is not yet clear. Duran-Reynals has shown that vaccine virus injected by any route localizes where testicle extract has been injected intracutaneously, and an extensive lesion results. Other investigators (5, 6, 7, 8) as well as ourselves (9) have proved that extracts of testicle and kidney greatly enhance cell multiplication and growth *in vitro*. On the other hand, the important rôle played by injury and by repair in the onset or localization of infection is well known. It is possible that the Reynals factor from testicle and kidney extracts either acts on the host cells to alter them directly, or else that they are so altered in the processes of injury and repair that they become a more suitable medium for infection. Work is now being carried out on the effects of these extracts on bacteria themselves.

The interfering action of lymphoid tissue on normal and pathological cells may be here recalled (10) in connection with the fact that spleen extract does not enhance the lesions produced by staphylococci, and even in many cases tends to lessen the size of the lesion.

CONCLUSION

The addition of testicle extract to cultures of 20 different bacteria just prior to inoculation enhances their infectious activity to a high degree.

Kidney extracts enhance the infections of staphylococci to a less

ing the invasiveness of the virus and diminishing the period of incubation.

With vaccine virus, it was desired to find out (a) if it is possible to reproduce in rabbits the equivalent of postvaccinal encephalitis as it occurs in man (which Turnbull and McIntosh (9) believe they have accomplished even without the aid of the Reynals factor), and (b) in what manner the virus reaches the central nervous system after peripheral inoculation.

Methods and Materials¹

The viruses and the testicular extract were prepared under sterile conditions and were implanted in the tissues of rabbits or guinea pigs as follows:

Herpes Virus.—Fresh rabbit brain virus in 1:5 to 1:10 suspensions was used. 0.5 cc. of the suspension and 0.5 cc. of saline solution were inoculated endermically in one area of the shaved abdominal skin of rabbits and similar amounts of virus and testicular extract in another area. In guinea pigs, only 0.3 cc. of each of the materials was injected.

Vesicular Stomatitis Virus.—With all animals a 1:20 suspension of glycerolated, guinea pig pad virus was employed and 0.4 cc. of each of the materials was injected in a manner similar to that used with herpes virus.

Borna Disease Virus.—The type of virus inoculated varied in different experiments: 0.25 cc. of a 1:5 suspension of fresh rabbit brain virus, or 0.4 cc. of 40 per cent suspension of glycerolated brain, or 0.001 to 0.004 gm. of dried cerebral tissue suspended in 0.4 cc. Ringer's solution was injected intracerebrally in rabbits as controls, while 0.25 cc. of testicular extract plus an equal amount of virus suspension was inoculated into the test animals.

Vaccine Virus.—The virus employed was the neurovaccine strain obtained several years ago from Professor Levaditi and at intervals propagated in rabbits' testicles or brain, or preserved in glycerol. 0.4 cc. of a 1:5 to 1:10 suspension of fresh brain virus and 0.4 cc. of a 1:5 dilution of testicular extract were injected. One group of rabbits were inoculated in the sciatic nerve, and another in the skin. Of the first group, the nerve of some animals was severed, a 2 cm. section resected and inoculation made about 4 cm. below the cut end. Control tests consisted of substituting broth or saline solution for the extract, or of inoculating the Reynals factor alone.

The results of the experiments may be summarized as follows:

¹ All operations were done under full ether anesthesia.

THE EFFECT OF TESTICULAR EXTRACT ON
FILTERABLE VIRUSES

BY DONALD C. HOFFMAN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Duran-Reynals (1, 2) has shown that the extract from normal testicles of animals can enhance remarkably the lesions produced in the skin by vaccine virus. In view of the fact that Pijoan (3) has found that the extract also promotes the pathogenic action of 20 kinds of ordinary bacteria, an attempt has been made to study the possible enhancing effect of the agent, called by Ledingham and Barratt (4) the Reynals factor, on the action of four different filterable viruses, namely, those of herpes, vesicular stomatitis, Borna disease, and vaccinia.

Herpes virus was chosen because two strains were available: one (H. F.) designated by Flexner "strong" (5), and the other (F.) "weak" (6). In the rabbit the strong virus has high and the weak low neurotropic activity; each strain has a moderate dermatotropic action. A special object, therefore, in using this material was to note whether, by the aid of the Reynals factor, a weak strain could be made strong and a strong virus more active.

In employing the virus of vesicular stomatitis of horses, we depended on the facts that this agent fails to infect any other part of the skin of guinea pigs than the hairless pads, and that the rabbit is generally much less susceptible than the guinea pig (7). Hence it was also attempted, in this instance, to determine whether the Reynals factor could so increase the action of the virus as to infect (a) ordinarily insusceptible tissue, *e.g.*, the abdominal skin of the guinea pig, and (b) a relatively resistant animal, the rabbit.

In the case of Borna disease, the virus of which was selected because of the unusually long incubation period of the affection—3 weeks or even much longer (8)—the study concerned the possibility of increas-

Vesicular Stomatitis Virus

As a preliminary experiment, two guinea pigs were each inoculated, in one posterior pad with vesicular stomatitis virus, and in the other with virus mixed with testicular extract. The pad first mentioned showed, after 48 hours, moderate vesicular dermatitis, the vesicles of which yielded a small amount of clear exudate. The other exhibited, after 24 hours, edema and, a day later, a prodigious vesicle covering the entire pad; ultimately the skin was shed in a complete, massive cast. The Reynals factor is, therefore, capable of enhancing the effects of the virus in guinea pig pads, an ordinarily sensitive tissue.

Twenty-eight guinea pigs were injected intracutaneously in the shaved abdominal skin, in one area with the virus itself, in another with virus mixed with testicular extract. In agreement with the known relative insensitiveness of the abdominal skin, the areas containing virus alone showed, from 48 to 72 hours after injection, no reaction in five animals, and a pin-head sized nodule in the remaining 23. Of these last, only seven animals yielded a drop of exudate on pricking the small nodules, which after a day or two rapidly subsided. On the other hand, those skin areas harboring virus and testicular extract all exhibited, after 24 hours, extensive vesicular dermatitis, yielding a plentiful supply of clear, serous exudate which in turn proved active in the pads of normal guinea pigs. The site of inflammation, characterized by hyperemia, edema, vesiculation, and induration, extended to an area of 3 cm. in diameter, and did not begin to show signs of healing until after 5 to 7 days. Microscopic study of the tissues removed from 14 of the animals revealed no specific changes in the sites inoculated with virus alone, but a varying degree of edema, cellular exudation, vesicle formation, with characteristic intranuclear inclusion bodies in the epidermis and subcutaneous tissue in the areas containing virus mixed with the Reynals factor.

Eight rabbits received endermically in the shaved abdominal skin virus alone. Four were unaffected and four showed only a minute, firm nodule at the site of inoculation. By way of contrast, an area of skin into which was injected a mixture of virus and extract exhibited, in seven of the eight animals, well-defined vesicular dermatitis. The clear fluid obtained from the vesicles was specifically active in guinea pig pads, and sections of the involved skin revealed lesions similar to those observed in guinea pigs.

In another experiment, Berkefeld V filtered testicular extract was used instead of the unfiltered material. In 13 rabbits and guinea pigs enhancing effects were observed which were not different from those of the unfiltered Reynals factor. An extract of guinea pig pads, prepared after the manner of testicular material (1) was substituted for the latter in another series of six animals: only a slight exaltation of the virus effects was noted in guinea pigs and practically none in rabbits.

The filtered or unfiltered Reynals factor promotes the action of vesicular stomatitis virus. Furthermore, by means of testicular ex-

Herpes Virus

Nine rabbits and five guinea pigs were inoculated endermically with the strong (H.F.) strain of herpes virus and eleven with the weak (F.) strain. In each of the nine rabbits the area containing the herpes agent mixed with the Reynals factor showed, usually after 48 hours, an extensive hyperemia and edema which was promptly followed by well-defined vesiculation. Thereafter considerable induration occurred with drying and scaling of the vesicles. The lesions endured until the death of the animal, usually about 10 days after inoculation. In the area containing the virus alone, simple vesiculation was noted which soon became nodular and showed signs of early healing; the extent of the dermatitis was only one-fourth to one-half that caused by virus mixed with testicular extract. Moreover all nine rabbits exhibited on about the fourth, and occasionally as late as the seventh day, signs of encephalitis. Death ensued from the fifth to the eighteenth day (average 10.3 days) after inoculation.

In the guinea pig still more striking differences were discernible in the lesions induced by the H. F. virus alone and by this material mixed with the Reynals factor. Clinically, the changes were respectively similar to those noted in the rabbit; quantitatively, the dermatitis caused by the virus in association with the Reynals factor covered an area 4 to 10 times larger than that produced by the virus alone.

Of eleven rabbits inoculated endermically with the weak (F.) strain of herpes virus, three revealed, in the areas containing virus alone, no lesions and eight small nodules or single vesicles, 0.5 to 1 cm. in diameter, which rapidly subsided. On the other hand, all eleven animals exhibited at the site of inoculation of virus mixed with the Reynals factor, reactions of hyperemia, vesiculation, induration, and in some instances marked ulceration. Indeed, the lesions covered a space from 6 to 20 times larger than the area of dermatitis induced by the virus itself. Of the eleven rabbits, ten developed encephalitis after an average incubation period of about 6 days and nine of the ten died 11 days after inoculation. These results are in striking contrast to those obtained from the action of the F. strain alone, as described by Flexner (10) and Gay and Holden (11), who found that the F. virus produces in rabbits only a non-fatal vesicular dermatitis.

To summarize the experiments with the two strains of herpes virus: the effect of the Reynals factor on both was to intensify their pathogenicity in the skin and brain. In other words, a strong strain has been made still more active, and a weak, dermatropic strain has acquired the properties of a strong virus, as characterized by its more intensive action in the skin and by its marked and usually fatal effect in the brain.

also occurred. In one rabbit the changes described could be followed throughout the sciatic nerve to the spinal cord and thence to the brain. Furthermore, the brains of five and the spinal cords of two rabbits were examined and appreciable amounts of virus were found in all.

Fifteen rabbits were submitted to the influence of testicular extract injected into the brain or spinal cord either at the time of intraneural inoculation of vaccine virus, or 2 days later. Thirteen of the animals revealed clinical reactions indicating encephalitis; seven yielded active virus from the brain or cord or both and, of twelve rabbits examined, eleven showed definite microscopic evidence of encephalitis. Comparing the results of this series with those already given, it may be stated that the animals treated with the Reynals factor exhibit more pronounced vaccinal lesions than those not so treated. The differences are, however, in degree rather than in kind.²

In another series of tests in which seven rabbits were employed, the virus either alone or mixed with the Reynals factor was injected into the cut and resected sciatic nerve, as already indicated. It was found that blocking the path in the nerve obstructs the passage of the virus and diverts it from nerve extension to blood distribution.

The next experiments with neurovaccine virus concerned its endermic inoculation in rabbits.

Intracutaneous Inoculation.—It is known that the injection of neurovaccine virus into the skin of rabbits induces no outward signs of cerebral involvement, nor any distinct histopathological changes of encephalitis. Virus, however, can be recovered from the brain (12). Under the influence of the Reynals factor, on the other hand, a definite neurotropism is demonstrable after endermic inoculation of the vaccine virus, as is disclosed by the results on 16 rabbits. In six of these animals testicular extract was injected admixed with the virus, and in the remainder it was injected independently into the brain, in two cases at the time of endermic inoculation of the virus, and in eight after a lapse of 2 days. Seven of the 16 rabbits exhibited clinical signs of cerebral involvement, and seven died from vaccinal infection. Virus was recovered from the brain of 13 animals—all that were examined; the spinal cord of seven of these was tested, and in five cases was found to contain the vaccinal incitant. The central nervous system of twelve rabbits was studied microscopically. Of these animals, eleven revealed characteristic lesions of vaccinal encephalitis, together with, in four instances, changes in the spinal cord also. The lesions in the nervous system were similar to but milder than those occurring after intraneural inoculation. Such effects as necrosis and destruction of neurones were not seen and the polymorphonuclear cell exudation was relatively less marked.

² We are indebted for some of the observations recorded to the kindness of Dr. C. P. Rhoads.

tract, the virus can be activated not only in relatively resistant tissues of a susceptible host, e.g., in the abdominal skin of guinea pigs, but also in similar tissues of a relatively insusceptible animal, the rabbit.

Borna Disease Virus

In view of the fact that the onset of infection by Borna disease virus is not clearly defined in rabbits, the interval of time between implantation of the virus and death from its specific effects was accepted as a measure of its activity.

Twelve rabbits were inoculated intracerebrally with 0.4 cc. of material containing the virus. The average time between inoculation and death from specific infection was 53.7 days. Eleven animals were similarly injected with only 0.25 cc. of virus suspension and with a similar amount of the Reynals factor. In this case the average period of survival was 32 days.

In this experiment, the pathogenic action of the virus was markedly increased by the Reynals factor.

Vaccine Virus

The first experiments related to the inoculation of neurovaccine virus into the sciatic nerve of rabbits.

Intraneural Inoculation.—Of seven animals, as controls, which were injected intraneurally with neurovaccine virus, five showed from the second to the seventh day after inoculation varying degrees of clinical reactions indicative of encephalitis. They developed fever (40.1 to 41°C.), hypersensitiveness, ataxia, and, in one case, complete paralysis of the posterior extremities, gnashing, salivation, urine retention, and opisthotonos. Three of the animals died on the third to fifth day as a result of vaccinal infection. The others were etherized on the seventh and eighth days for microscopic study of the nervous system.

The histopathological changes were, first, those of meningo-encephalitis. Edema of the pia-arachnoid with infiltration of monocytes and of relatively fewer polymorphonuclear leucocytes was observed. In most instances the meningitis was localized to certain areas. The cortex occasionally showed edema, with diffuse infiltration of monocytes, of varying degree in the different animals. Perivascular infiltration of monocytes, of varying degree in the different animals. Perivascular inflammation was paralleled by that following the inoculation of vaccine virus directly into the brain. Secondly, a myelitis was present. The changes in the spinal cord of two rabbits consisted, in the membranes, of thickening by edema and cellular infiltration and, in the white matter, of areas of localized necrosis, perivascular infiltration, and occasional small hemorrhages. The infiltrating cells here were monocytes and a few polymorphonuclear cells. Thirdly, a neuritis occurred: the sheath of the sciatic nerve was thickened by edema and cellular infiltration. The nerve tissue was hyperemic; localized hemorrhages and cellular infiltration

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To summarize the results of experiments with neurovaccine virus, it appears that after peripheral inoculation the virus may extend by way of the peripheral nerve to the spinal cord and thence to the brain. It can therefore evoke definite disturbances of the nervous system, neuritis, myelitis, and encephalitis, which are detectable clinically and pathologically.³ Moreover, virus can be recovered in appreciable amounts from the nervous tissues. While all these effects arise from vaccine virus alone, they are particularly noticeable when the virulence of the incitant is promoted by means of the conjoint action of the Reynals factor. A clinical and pathological process reproducing postvaccinal encephalitis as it occurs in man was not secured by these experiments.

CONCLUSIONS

The Reynals factor promotes the pathogenic action of the viruses of herpes, vesicular stomatitis of horses, Borna disease, and vaccinia. The heightening of virulence is revealed in various ways. The effects of the viruses may be accentuated; or a weak strain converted into a strong one, as in the case of the F. strain of herpes virus; or the power acquired to infect resistant species or tissues, as, *e.g.*, rabbits and the abdominal skin of guinea pigs, with acute vesicular stomatitis. The Reynals factor should serve as an important agent in the study of filterable viruses.

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³ The lesions induced by neurovaccine virus³ are distinguishable from those of spontaneous encephalitis and myelitis in rabbits. The reactions to the vaccinal incitant are primarily of an acute inflammatory type, involving, first, the coverings of the brain or cord, then the perivascular spaces, and finally the substance of the cortex or of the cord. The characteristic changes include edema and a varying degree of polymorphonuclear cell exudation.

Phenomena of Tuberculosis as

Phenomena of the disease		Production of lesions		M/L index in blood	Rate of growth of bacilli	Estimated proportion of dead to living bacilli	C vir b fro
		Tubercles	Non-specific connective tissue				
Relation of chemical fractions from the bacilli to these phenomena		Phosphatide	Wax and protein	Relation of phosphatide to anti-phosphatide	?	?	
Animal	Type of bacillus						
Fowls	Avian S (Petroff)	+		Reversed ++++	++++	Small	
	Avian R (Petroff)	+++		Slight change	++	Average	
Guinea pigs	H ₃₇	+++		Reversed ++	Average	Average	
Rabbits	B ₁ and frac- tions of H ₃₇	++++	++++ ++	Reversed ++++	Average	Large	
Monkey		++++	++++	Reversed ++++	++++	Large	
Rat		++++				Extreme	

Along the upper transverse line of the table are listed the phenomena of the disease itself and normal and tuberculous animals as far as they are known. The lower part of the chart records the results of the chemical analysis of the fractions from the bacilli.

A STUDY OF THE TOXIC PROPERTIES OF TUBERCULO- PROTEINS AND POLYSACCHARIDES

By FLORENCE R. SABIN, M.D., FRANKLIN R. MILLER, M.D., CHARLES A.
DOAN, M.D., AND BRUCE K. WISEMAN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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It was reported by White in 1928 (1) and confirmed by Sabin, Doan, and Forkner (2, 3) that a polysaccharide isolated from the tubercle bacillus by Anderson (4) was toxic for tuberculous guinea pigs.

With doses of 10 mg. the guinea pig either died within a few hours, in which case there was a precipitous fall in temperature; or it survived and showed only a temporary fall in temperature with subsequent rise above the normal level. There was also a change in the blood cells characterized by a rise in the neutrophilic leucocytes and a fall in lymphocytes.

Similar phenomena follow the administration of tuberculo-protein. Since the polysaccharide, as prepared, has a certain nitrogen content, and since all the protein preparations contain carbohydrate, one must resort to a biological titration in order to determine responsibility for the intoxication. By giving the polysaccharide and the protein to tuberculous animals, in decreasing doses, we have ascertained that the temperature reaction is due to the protein, since amounts of polysaccharide which do not contain as much nitrogen as is present in a temperature-altering dose of the protein fail to elicit the change.

In the course of our testing of fractions from tubercle bacilli, it has become clear that we must consider not only fractions from different types of bacilli, according to the plan of the Research Committee of the National Tuberculosis Association (5), but also the varying manifestations of the disease in different species of animals. To show the relations of these different factors we have drawn up an outline (Table I). The attempt at any quantitative comparative estimation of the factors of the disease must be considered as only an approximation, subject to marked variations in individual animals. For the

data on the temperature reactions in the monkey we are indebted to Dr. G. P. Berry whose work will soon be published.

The phase of the subject upon which we have done the most intensive work (3) is that of the chemical factors causing the production of new tissue. Both lipoids and proteins in repeated doses have given rise in rabbits to the production of new connective tissue cells, but a phosphatide designated A-3 by Anderson (6) has proved to be so specific in the production of epithelioid cells and epithelioid giant cells, the essential elements of the tubercle, that we may speak of this element in the disease as the phosphatide reaction. In the tuberculous animal the phosphatide is probably liberated only from the bodies of dead bacilli. The amount of the reaction to the phosphatide is governed by two different factors, first, by the number of dead bacilli, which can only be estimated; and second, by the amount of antiphosphatide produced in the disease (7, 8, 9).

The most striking new material shown in the table is represented by the preliminary analysis of the effects of the dissociation of the avian strain of bacilli by Petroff (10, 11, 12). These two strains were given to us by Dr. Petroff for pathological studies. As shown in Table I, the two dissociated strains of the bacilli produce different types of tuberculosis, that is, two different combinations of the manifestations of the disease. Roosters with the avian S strain had no tubercles large enough to be seen with the unaided eye, but under the microscope there were numerous tiny clumps of pure epithelioid cells, containing very large numbers of tubercle bacilli. Smears stained for tubercle bacilli had too many organisms per oil immersion field to be counted. From the small phosphatide reaction, we estimate that there had been a relatively slight death rate of bacilli. The animals had negative skin tests but became extremely emaciated.

With the avian R strain, on the other hand, the skin test was marked and the loss in weight only nominal, while tubercles were comparable in size to those seen in rabbits. Such striking differences in reaction to bacterial dissociation demonstrate the significance of this method in the further study of tuberculosis. A comparison of the reaction with the avian S in roosters with the disease in rats is also interesting. Both show a large number of bacilli, but in the case of the rat, we estimate an increased death rate of bacilli from the extent of the phosphatide reaction, the rat finally dying from the amount of epithelioid cells in the lungs rather than from any toxic effect. The detailed account of the pathological differences with dissociated strains will be published subsequently.

The data in Table I suggest, for the further testing of chemical fractions, the selection of animals showing certain extreme reactions, such as the loss of weight after the Petroff avian S in roosters, or the caseation in monkeys. The hypersensitivity of the guinea pig to tuberculo-protein has been the reason that this animal has been used so extensively for the study of allergy. Through the studies reported

LE I

to Chemical Fractions from the Bacilli

Acute and subacute or chronic	Loss in weight	Acute toxic death (experimental)	Allergy (skin test)	Caseation	Temperature	Hemorrhage
?	?	Protein and polysaccharide	Protein	Probably protein	Protein	Protein
Acute	++++	Protein 10-2.5 mg. and polysaccharide	±	—	Protein up to .0001 mg.	++++ Lymph nodes
Subacute	++		++++	++		
Subacute and chronic	Minimal		++++	+++		
Acute or chronic	+++		+	++ Slight with phosphatide in normal	Sensitive to O. T.	++
Acute	±		±	++++		++++
Chronic	+		—	— Epithelioids filled with fat		++

the second line are recorded the effects of chemical fractions isolated from tubercle bacilli on varying manifestations of the disease in animals, viewed in the light of studies with chemical

here, it seems probable that the temperature in tuberculosis as indicated in Table I, is related to proteins liberated from the bacilli. The tuberculo-proteins give a rise in temperature in the normal animal but the reaction is increased in the sensitized animal.

The two types of temperature curves, one the fall which precedes death, and the other the reaction from which the animal recovers, are shown in Charts 1 to 7 for both proteins and polysaccharides. The results of the titrations of these two substances in tuberculous guinea pigs are shown in Tables II and III.

Most of the experiments with the protein have been made with a preparation from the H. K. Mulford Company, designated MA-100, which gives an active skin test in tuberculous guinea pigs in doses of 0.0001 mg. One guinea pig received the Protein 304 and another an alkali-soluble protein, both prepared from the bacilli by Johnson, Coghill and Renfrew (13, 14).

Four preparations of tuberculo-polysaccharides have been used. First, a sugar isolated by Anderson (15) from the water separated from the ether-alcohol extraction of lipoids from H-37, and designated A-8; second, an analogous sugar from the bovine strain; third, preparations from the Bacilli H-37, prepared by Heidelberg, Hbg 511 and 514; and fourth, similar preparations made by the H. K. Mulford Company, MB-200 (16). The guinea pigs used in these experiments were all tested with tuberculin (0.02 cc. O. T. Saranac) and were negative before the inoculation with tubercle bacilli. They all received 1,000,000 counted organisms (1/50 mg.) of a 10 day culture of human Strain H-37 obtained from Dr. S. A. Petroff. They were inoculated either intraperitoneally or subcutaneously in the groin, as shown in Tables II and III. The skin test was positive to O. T. in each instance before the injections of protein and polysaccharide were begun.

The rabbits were given 0.5 mg. of the Strain B-1 undissociated, intravenously from a 13 day culture. The experiments with the proteins and the polysaccharides were begun about 6 weeks after inoculation and were continued through 4 months. One (R 1344) of the 20 guinea pigs of the first series (inoculated Feb. 14, 1930), died 18 weeks after inoculation without having received any injection, and showed caseous inguinal lymph nodes and extensive tuberculosis of liver, spleen, omentum and lungs. Other animals that died following the injection of both protein and polysaccharide have shown marked lesions of the liver and spleen. In every case, with one exception, the routine was to take a rectal temperature first, then the specimens for the blood count and then give the injection. The exception was the first experiment on Chart 3, Guinea pig R 1323, in which the injection was given first. The amount of fluid injected was in each instance 1 cc. The polysaccharide and the water-soluble Protein 304 were dissolved in freshly distilled water. The alkali-soluble protein was dissolved in distilled water and then

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	30	2/14/30	Alkali	3/25/30	Intra-	+4.5°	5883	3330	1554	5200	9152	5616	19712	1888	507	Survival
	R 1342	(s.c.)	soluble protein (John- son)													
0.1	16	2/14/30	Mulford	4/16/30	i.p.	-1.8° +2.2° -3.9°	4140	5934	1380	2782	1575	735	8532	756	1404	"
0.02	3	2/14/30	"	4/21/30	i.p.	-.8° +3° -5°	1768	1224	340	2366	1820	364	3960	176	110	"
0.01	30	2/14/30	"	4/22/30	i.p.	-1.5° +3.4°	5883	3330	1554	12198	14766	2568	19276	2254	483	"
0.001	31	2/14/30	"	4/22/30	i.p.	+2.9°	6732	2856	408	5016	5700	684	11808	1065	1152	"
	R 1343	(s.c.)														
	31	2/14/30	"	3/25/30	Intra-	+3.2°	6732	2856	408	12243	6237	4389	14700	2952	980	"
	R 1394	(s.c.)			testic- ular											
	30	2/14/30	"	4/23/30	i.p.	+2.5°	—	—	—	1527	1560	162	4972	526	396	"
	R 1342	(s.c.)		4/25/30	i.p.	- .7° +3.7°	5883	3330	1554	5475	9417	3285	16942	1576	985	"
	7	2/14/30	"			Negative	1260	714	126	4380	1440	180	7296	498	1462	"
	R 1319	(i.p.)		5/ 1/30	Intra- dermal	-1.3° +1.4°										

* These are serial numbers of the work of the department covering a term of years.

A more gradual fall in temperature, 7° in 7 hours, is shown in Chart 2, of Guinea pig R 1321, which received 5 mg. of the Protein MA-100 intraperitoneally.

The animal died in 16 hours after the injection. The postmortem examination showed extensive involvement of omentum, spleen, liver, and inguinal lymph nodes; there were a few tubercles in the lung. Fresh hemorrhages were not apparent at autopsy, but in sections there was marked congestion of the vessels of the lung and some hemorrhage into the tubercles. It will be noted in the chart that there was a fall in neutrophilic leucocytes, lymphocytes, and monocytes during the 7 hours that counts were made. All but one of the animals, R 1399, that died within 24 hours after the injection, had the marked fall in temperature.

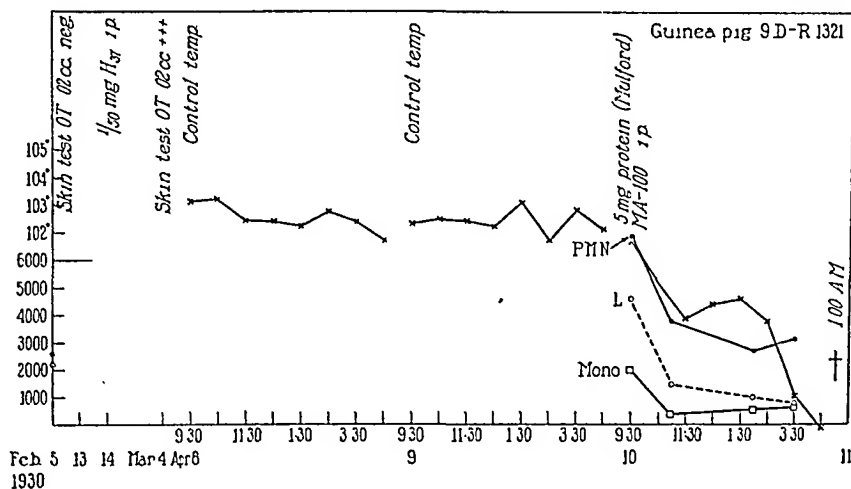


CHART 2

In this instance the changes in temperature were but slight; there was a drop of a degree and then a rise to the original level; the temperature had begun to fall when last taken and the animal died in the night. All of the animals that showed the fall in temperature were obviously quite sick, showing a marked toxic effect of the protein. The other animal that died after protein showed the same extensive involvement of liver, omentum, spleen, and lymph nodes. The lungs had a few tubercles and were markedly congested and in one case (R 1406) hemorrhagic.

The polysaccharides had also a certain killing power in sensitized guinea pigs, though it was by no means as consistently related to dosage as with the protein. In the original studies of Sabin, Doan, and Forkner (2, 3) six tuberculous guinea pigs received 10 mg. of the Polysaccharide A-8 intraperitoneally; two died within a few hours, two during the night after the experiment, while the others survived.

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enough 1 per cent NaOH added to make the solution neutral to litmus. The Mulford Protein MA-100 was received in sterile salt solution, 10 mg. per cubic centimeter, and it was diluted with freshly made, sterile salt solution.

Killing Power of Tuberculo-Proteins and Polysaccharides in Tuberculous Guinea Pigs

As is shown in Table II, all five tuberculous guinea pigs which received from 10 to 2.5 mg. of the protein died within 24 hours; and all receiving less than 2.5 mg., seventeen injections in all, survived. Thus the minimal lethal dose for this protein lies between 2.5 and 1 mg.

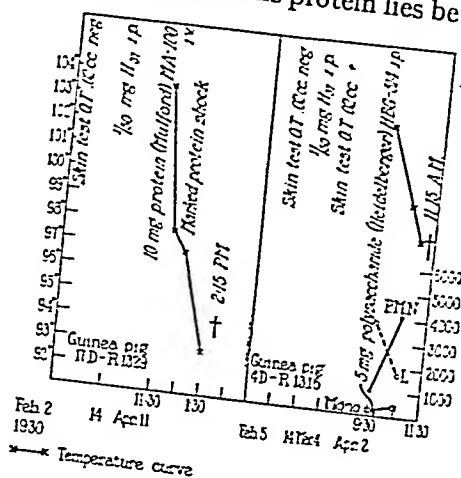


CHART 1

in tuberculous guinea pigs. A report of the killing power of protein prepared from the filtrate has been given by Seibert (17). In Chart 1 is shown the type of temperature curve following a lethal dose of the protein, for Guinea pig R 1329.

The injection was intravenous; the animal showed irregular and rapid breathing within 2 minutes of the injection. Subsequently there was involuntary defecation. The temperature fell precipitously 10°. The irregular breathing continued until death 4½ hours later. The postmortem examination showed the omentum, spleen, and liver massively involved with tuberculosis; the lymph nodes were markedly caseous; the lungs had a moderate number of tubercles. There were numerous fresh hemorrhages especially marked around the tuberculous lesions in the omentum and in the lung. The lungs were markedly congested.

The polysaccharides in the present series did not show as marked a killing power for guinea pigs.

As shown in Table III, one guinea pig, R 1324, died in 4½ hours after an intraperitoneal injection of 20 mg. of the A-8. The liver, spleen, omentum, and lymph nodes were only moderately involved; the lungs were markedly congested; but no hemorrhages were found in sections. This animal had survived two injections on the 2 preceding days of 10 mg. each of this polysaccharide. None of the animals receiving 10 mg. died; but R 1316 which had an intraperitoneal dose of 5 mg. of the Heidelberg polysaccharide died in 1½ hours, the fall in temperature and the changes in the blood cells being shown in Chart 1. At post-mortem there was extensive involvement of liver, omentum, and spleen. The lungs showed many tubercles, but were only moderately congested and had no hemorrhages. It is important to consider whether there was sufficient protein in this dose of the polysaccharide to account for the killing power and for the fall in temperature. The problem with the polysaccharide is complicated through not knowing the actual state of the nitrogenous contamination or its potency. Dr. Heidelberg found the nitrogen content of this sugar to be 0.34 per cent, so that if all of the nitrogen were calculated as protein, 1 mg. of polysaccharide would contain 0.021 mg. of protein; and 5 mg. would have only 0.105 mg. protein, which is below the minimal lethal dose of the protein whose potency we have been testing. On this basis it would take 100 mg. of the polysaccharide to contain 2.1 mg. of protein, 2.5 mg. having killed a guinea pig (Table II).

The question of a possible killing power of the polysaccharide here employed needs further study, with a larger series of experiments; but these experiments suggest that it either has a certain killing power in sensitized animals or it may enhance the killing power of a dose of protein too small to kill by itself. It should be stated that the manner of death is exactly like that of the lethal action of the protein.

In relation to the fall in temperature, on the other hand, it is interesting to note that the animal (Guinea pig R 1315, Table II) which received 0.1 mg. of the protein, the computed amount in the 5 mg. of polysaccharide, showed a final drop of 5° in temperature, so that if the fall in temperature in Guinea pig R 1316 (Table III) which succumbed to the polysaccharide was a direct effect of the substance injected rather than an indirect toxic effect on the animal, it might have been due to the protein introduced with the sugar.

It will be noted that one other animal which received 5 mg. of the sugar, R 1317, showed a fall in temperature and died 4 days later.

At the postmortem examination, extreme involvement of liver, spleen, omentum, and peripheral lymph nodes was found together with many tubercles in the lungs. There were extensive hemorrhages. They involved many of the lymph nodes and some of the organs, as well as the vessels in the skin and in the subserosal layers of the peritoneal lining. It may be that a sustained fall in temperature after the injection of either protein or polysaccharide indicates, even if the animal survives 24 hours, that it is not likely to survive the disease for many days.

In general the animals which died after both protein and polysaccharide had extensive involvement of the liver, spleen, omentum, and lymph nodes, and a moderate number of tubercles in the lungs. They all showed congestion of the vessels of the lung but no edema; most of them had hemorrhages around the tubercles in the lung and around the caseous areas in the omentum. The lymph nodes were congested.

Reaction to Sublethal Doses of Protein and Polysaccharide in Tuberculous Guinea Pigs

The animals which survived the injection of either of these substances did not show any marked effects except on the temperature and on the blood cells. They were not clinically ill.

The results were the same for both substances regardless of the route of injection, whether intravenous, intraperitoneal, or intratesticular. The type of the changes in the blood cells and the temperature are shown in Tables II and III; but the type of reaction is better indicated by curves as shown in Charts 3 to 5. A typical temperature reaction involved a preliminary fall lasting from $\frac{1}{2}$ to 2 hours, followed by a gradual rise of from 2 to 4.5° with a return to the original level in approximately 5 hours. The positive reaction was not only a change in temperature but a specific type of curve. The fall occurred at once after the injection; it was, however, sometimes much shortened or even suppressed, but the subsequent rise and return to the original level were constant. The sublethal reaction for the polysaccharide is shown by the first curve in Chart 3, R 1323, the animal having received 5 mg. of the Mulford polysaccharide intravenously. The injection was made before a preliminary temperature had been taken and no blood counts were taken. The animal had a slight chill but no other symptoms. Similar curves for the different proteins are shown in Chart 4, R 1342. The animal received an injection of 1 mg. of the alkali-soluble protein (Johnson) intratesticularly 5 weeks after inoculation. It was highly sensitized; the temperature fell slightly for an hour and then rose 4.5° by five o'clock. It was still above 103° the next morning, the original level having been 102°. It will be noted that the leucocytes rose in this instance while both lymphocytes and monocytes fell.

[illegible]

Biological Titration of the Tuberculo-Protein and Polysaccharide in Tuberculous Guinea Pigs.—Since the reaction of the temperature and of the blood cells to these two substances was the same and since each was contaminated with the other, it was necessary to see if one substance could give the reaction in such small doses as to exclude the other. The experiment with titrations of these two substances is shown in Tables II and III.

All but one of the guinea pigs receiving injections of the protein of from 1 to 0.0001 mg. gave the characteristic temperature curve and fall in lymphocytes. The one negative reaction was in Guinea pig R 1319, Chart 5, following the injection of 0.001 mg. of the Mulford protein given by the intradermal route. With the next dilution, containing 0.00001 mg. of protein, the reaction became varied; two were negative, one moderately and one markedly positive. This dosage thus approaches the limits of the reaction to the protein.

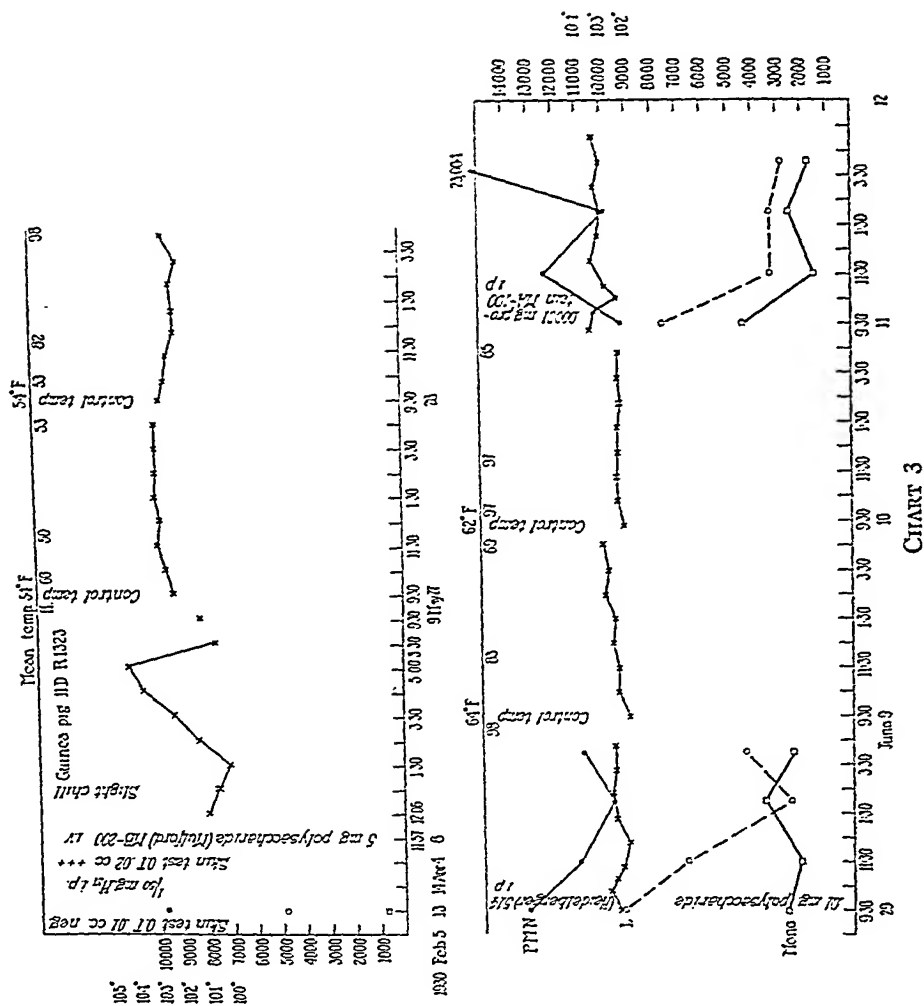
On the other hand, in Table III it is clear that the inconstant temperature reaction was obtained with the dose of 0.01 mg. of the polysaccharide; one animal was negative, a second showed a slight reaction and the third had a rise of 2.3° . On the basis that 1 mg. of the Heidelberger polysaccharide contains 0.021 mg. of protein or protein degradation products, 0.01 mg. would contain 0.00021 mg. of protein, which is twice the dose of protein which gave a consistently positive reaction.

There was probably enough protein, or its degradation products, to account for the temperature reaction in all of the experiments with the polysaccharides, provided the protein was in an active form.

Variations in Temperature Induced in Tuberculous Guinea Pigs by Protein and Polysaccharide

Certain interesting points can be made out in this connection.

In Chart 3 it will be noted that the guinea pig was highly sensitive to polysaccharide (protein) as shown by the first temperature reaction after inoculation with tubercle bacilli. During the months of May and June the control temperature curves were constant. These control periods are an important part of the experiment for they show that there is for the most part relatively little fluctuation in temperature from the disease itself. In only one animal of the entire series was there an afternoon temperature during one of these control periods; in this animal, R 1332, on one occasion the temperature was steady for the morning hours but rose suddenly 3.2° between one-thirty and two-thirty and then fell gradually to the original level. The cause of this rise was not determined. In Chart 3 it will be seen that the temperature remained normal both after 0.01 mg. of the Heidel-



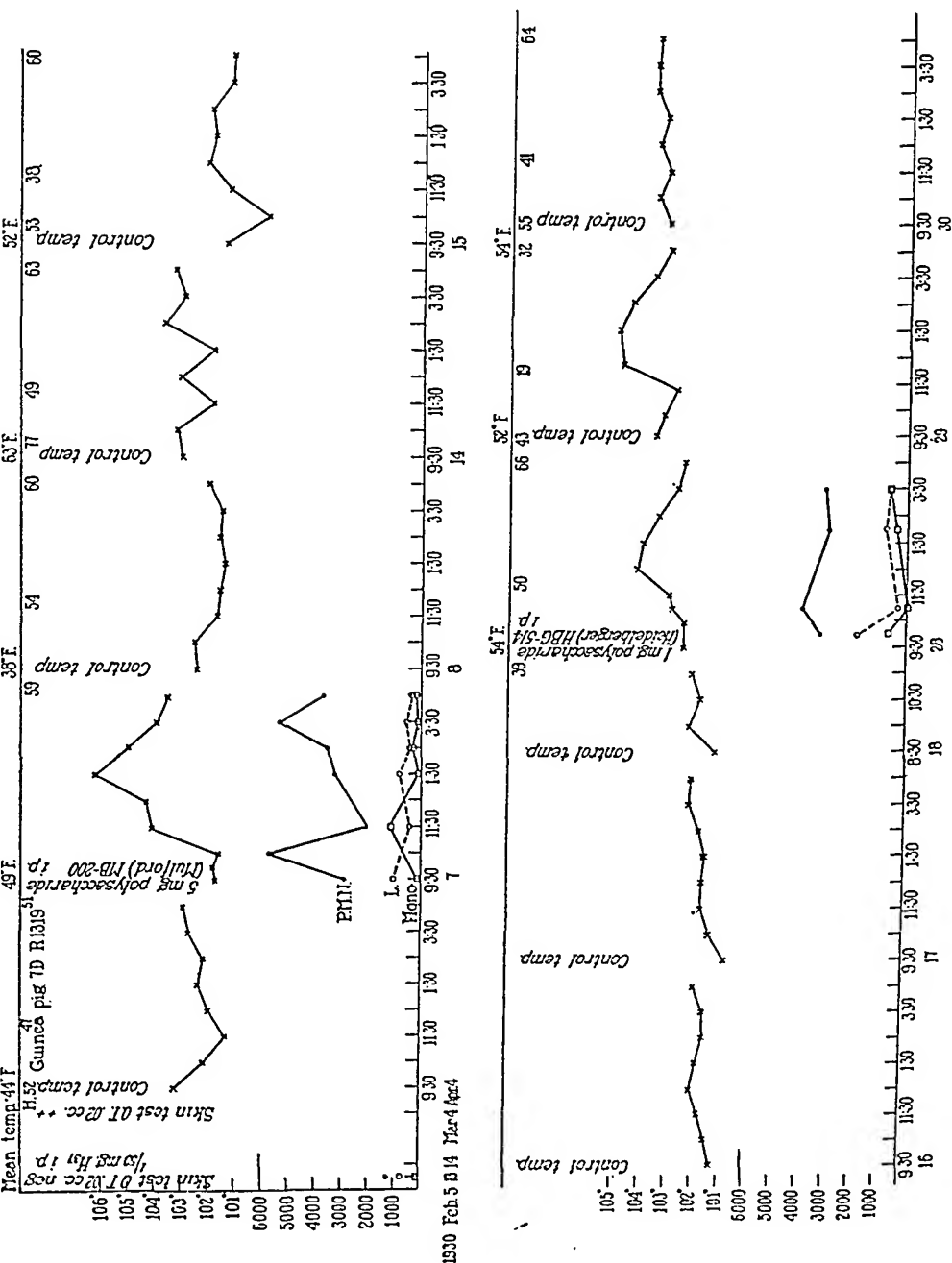


CHART 5—Continued on Next Page

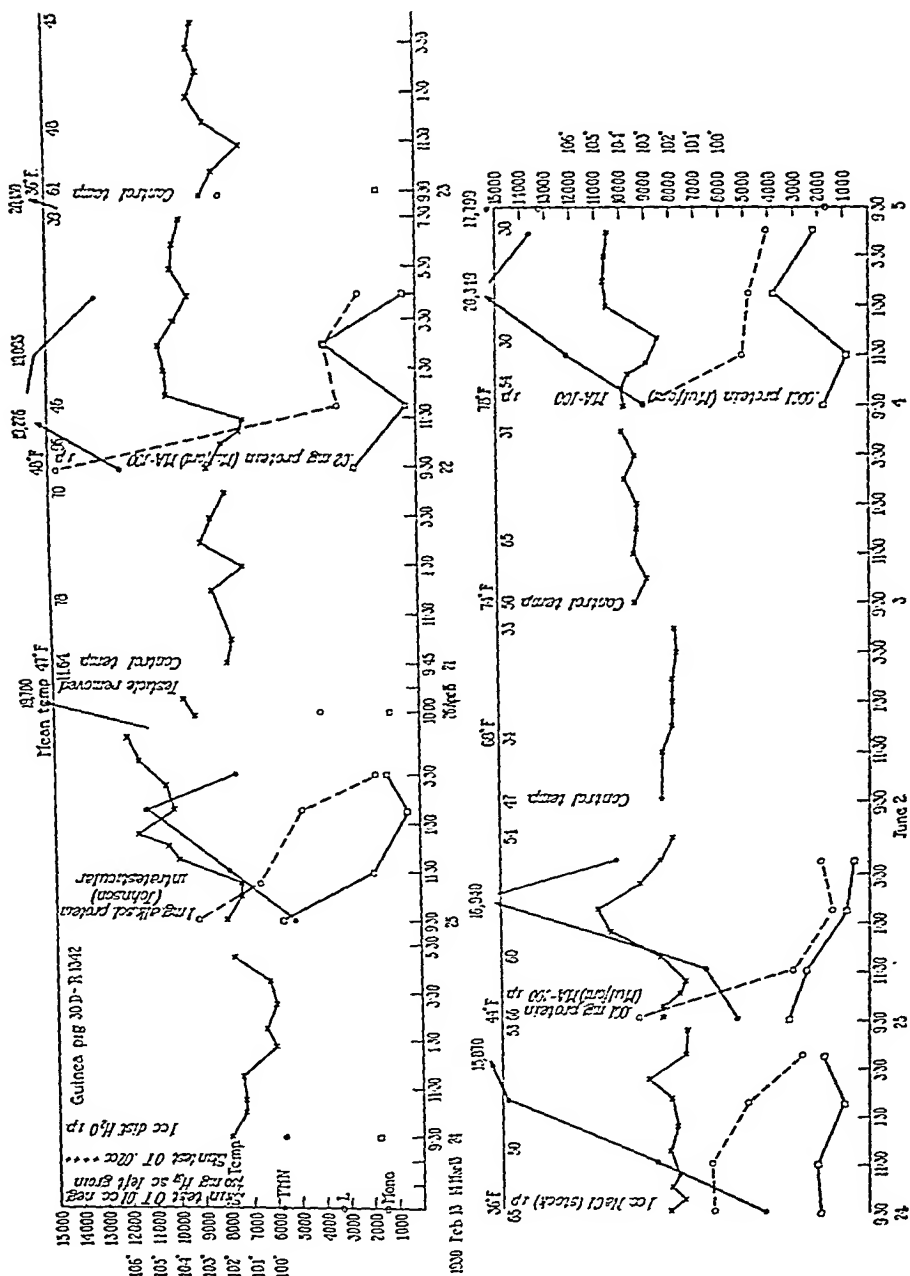


CHART 4

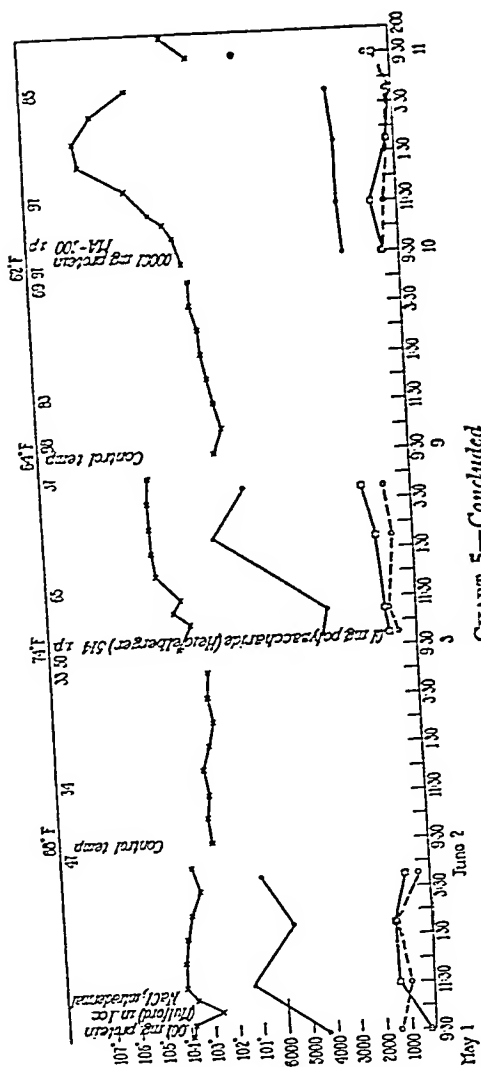
berger polysaccharide and after 0.00001 mg. of the Mulford protein; but on both of these days, the lymphocytes fell markedly and the neutrophilic leucocytes were irregular. Thus the reactions on the blood cells are not to be correlated with the change in temperature.

Chart 4 represents a long experiment with Guinea pig R 1342. The first experiment shows that there was no rise in temperature, but rather a fall after an injection of 1 cc. distilled water intraperitoneally. The water was freshly distilled from glass, boiled and used as soon as sufficiently cooled. A second control with distilled water in another guinea pig, R 1332, showed a steady reaction. The intratesticular route of injection was employed in R 1342 to study the tissue reaction as reported by Long (18). 21 days later the testis was removed and showed the marked degeneration described by Long.

By the twenty-first of April the control temperature was moderately steady, and the following day the animal gave a positive reaction to 0.02 mg. of the Mulford protein. The rise in leucocytes and the fall in lymphocytes were extreme; it is interesting that the lymphocytes had returned to 8000 cells per cubic millimeter by the next morning, though not to the original level. On the twenty-fourth of April a test was made with salt solution which had been sterilized and sealed several days previously; there was a slight rise in temperature in the afternoon which did not occur with freshly made salt solution. The rest of the experiment had to do with the minute doses of the protein, 0.001 and 0.0001 mg., with less reaction in temperature to the smaller dose.

In the intervening days, it will be noted that the level of the temperature of the guinea pig was a degree higher on the third of June than on the preceding day. This phenomenon has occurred both with normal and tuberculous guinea pigs. The mean temperatures for New York City, together with three readings for humidity, reported at 8 a.m., at 12 noon, and at 8 p.m., by the Weather Bureau, United States Department of Agriculture, are included in the charts. In Chart 4 it will be noted that with a rise of 6°F. between June 2 and 3, the temperature of the guinea pig was a degree higher on the second day. Comparing the humidities on June 2 and 3 it will be noted that on June 2 there was a fall of 13 points between 8 a.m. and noon, and on the next day a rise of about the same amount, namely 15 points, so that the change in temperature of the animal in this instance has run parallel with the change in atmospheric temperature rather than with the variation in humidity.

Chart 5 represents another long series of experiments. This guinea pig, R 1319, was markedly sensitive to an injection of 5 mg. of the Mulford polysaccharide on April 7, about 7 weeks after infection. The original control temperature was somewhat irregular, as frequently happens the first time temperatures are taken. The change in humidity was not great. It will be noted that the temperature of the guinea pig was entirely steady on the day after the injection, but on the 14th and 15th of April was irregular and ran nearly 2° lower on the 15th. There was a drop in atmospheric temperature of 11°F. The temperature of the animal became



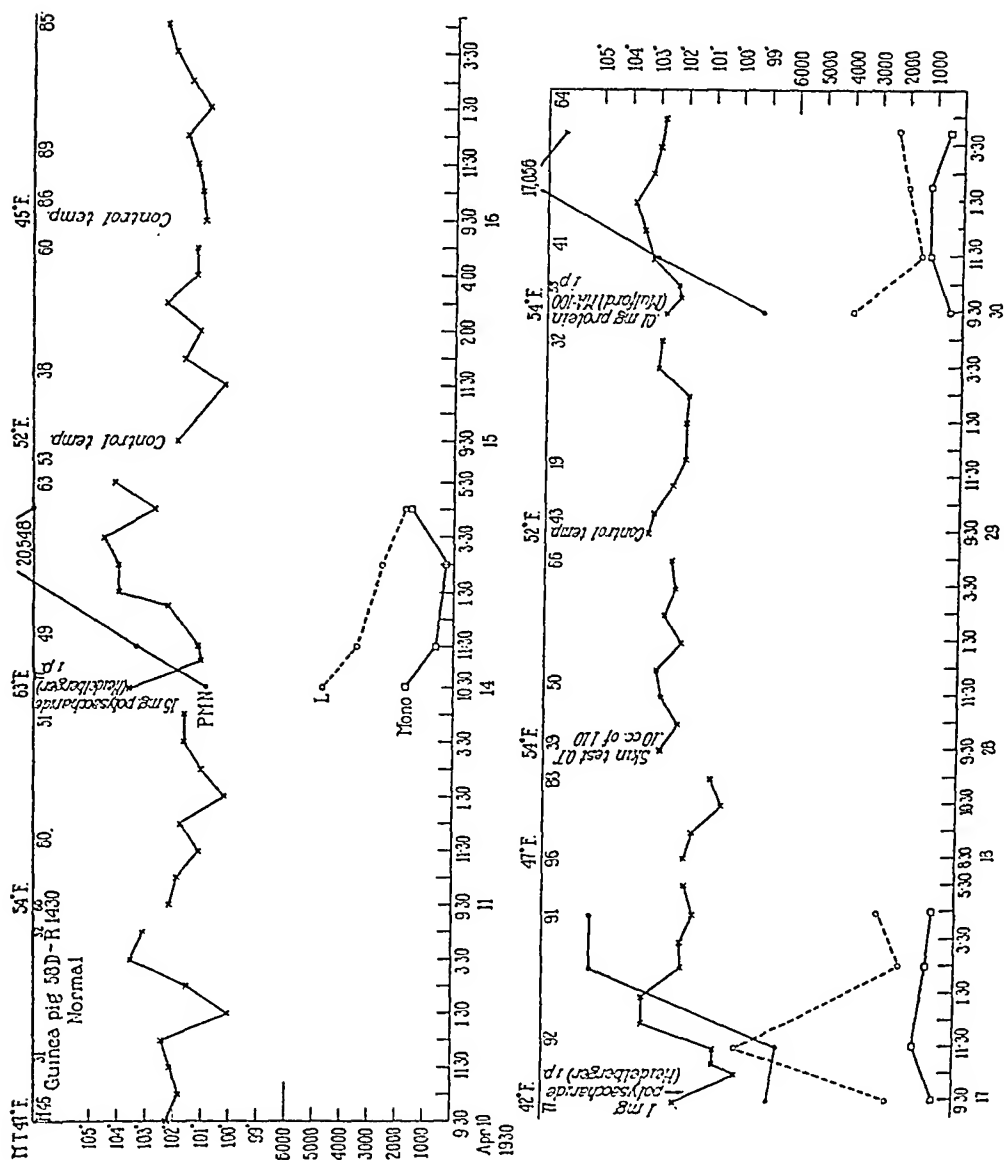


CHART 6

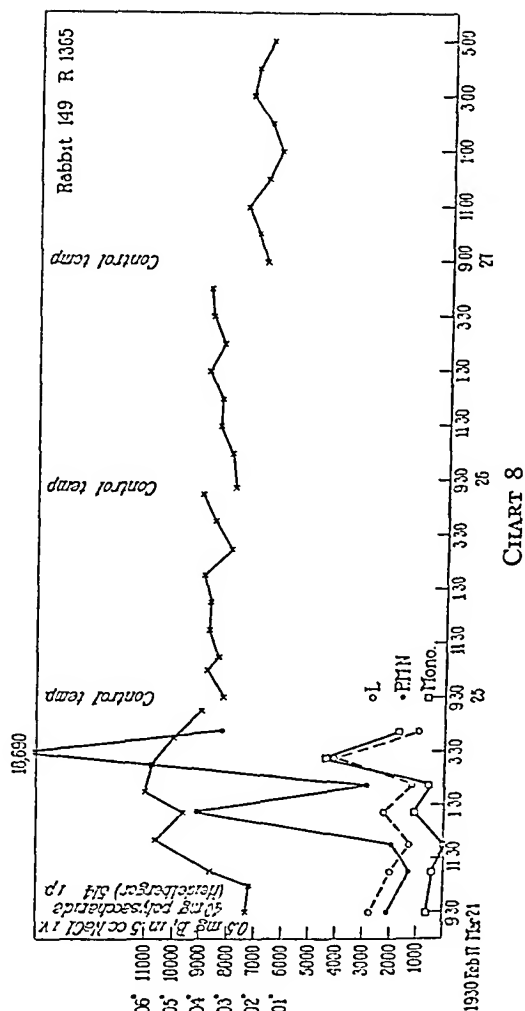
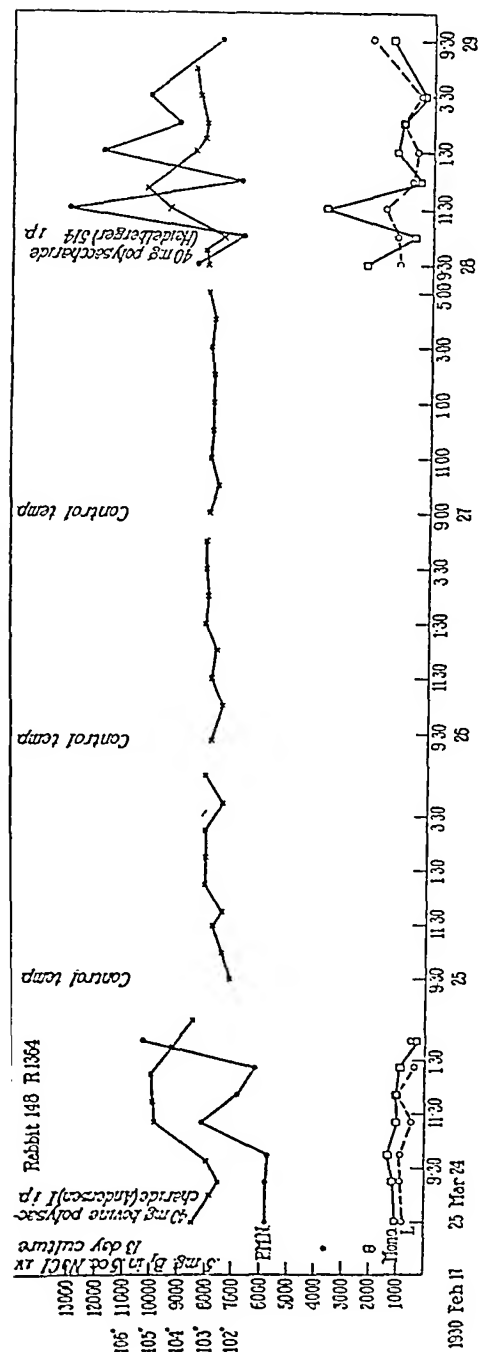
quite level again and on the 28th of April, it showed an average temperature reaction to 1 mg. of the Heidelberg polysaccharide. The next day there was a secondary rise, practically a duplicate of the curve of the preceding day with no change in the weather, and for 3 days the temperature of the animal was at a

TABLE IV

Effects of Tuberculo-Protein and Polysaccharide on Normal Guinea Pigs

Dose	Number of animal	Date of injection	Temperature after protein Ma-100	Fall of lymphocytes	Date of injection	Temperature after polysaccharide (Heidelberg)	Fall of lymphocytes
mg.				per cent			per cent
20	R 1433	6/ 6/30	+1.3°				
15	R 1430				4/14/30	-2.5° +3.5°	70
10	R 1432	6/ 6/30	+1.9°				
	R 1425				4/10/30	+1.7°	87
5	R 1425				4/ 8/30	-1° +1.5°	—
1	R 1430				4/17/30	-2° +3.4°	50
0.1	R 1430				5/22/30	-1.2° +1.2°	77
0.01	R 1430	4/30/30	+1.5°	70			
	R 1432				5/28/30	-2° +2.5°	61
	R 1433				5/29/30	+1°	65
	R 1436				6/ 3/30	-5° +1.6°	77
	R 1425				5/23/30	-1.3° +0.7°	69
0.001	R 1435	5/ 1/30	+1.6°	66			
	R 1432	5/ 2/30	+2.4°	63			
0.0001	R 1433	5/ 2/30	Negative	48			
	R 1436	5/ 1/30	-1.5° +1°	32			
0.00001	R 1434	5/ 2/30	Negative	76			

higher level than before the injection. We have seen no other secondary rise of this magnitude, but in three instances it has taken 3 days for the temperature to come down to the previous level (R 1323, Chart 3, after 5 mg. of Mulford polysaccharide; R 1331, (19) after 0.1 mg. of the Heidelberg polysaccharide; and Rabbit R 1365, Chart 8, after the Heidelberg polysaccharide). On May 1, R 1319, Chart 5, there was a negative reaction to an injection of 0.001 mg. of protein MA-100 given intradermally. On the third of June there was a slight



reaction to 0.01 mg. of the Heidelberg polysaccharide, but 7 days later the animal proved to be highly sensitive to the Mulford protein in a dilution of 10^{-8} . It will be noted also that the temperature of the guinea pig during the control period of June 9, was lower by 2°C . than the initial temperature of June 3. Between these 2 days the environment differed by 10°F . This guinea pig showed low lymphocytes throughout the experiment but they fell with every injection except after the 0.01 mg. of the polysaccharide.

Effects of Tuberculo-Protein and Polysaccharides on the Temperature of Normal Guinea Pigs

The results of experiments with two types of fractions are shown in Table IV and Chart 6.

For all of the work the Mulford Protein MA-100 and the Heidelberg Polysaccharide 514 were used. It will be seen that with the normal animal the rise of temperature was more moderate throughout than with the sensitized animal; the type of curve was, however, the same. It is likewise clear that the biological titres of polysaccharide and protein exhibit the same relationship in the normal guinea pig as in the sensitized; however the necessary amounts for normal animals are 10 to 100 times greater. For the polysaccharide, the reaction was either negative or slight with doses of 0.1 and 0.01 mg.; while with the protein the limit of reaction was 0.001 mg. and the dose of 0.0001 mg. was negative. In Chart 6 it will be noted that during the control periods the temperatures were more irregular but were not at a lower level than with the tuberculous animals. There were more of these irregular control temperatures in normal than in tuberculous guinea pigs.

Effects of Tuberculo-Proteins and Polysaccharides on Tuberculous and Normal Rabbits

Fewer experiments were made with rabbits than with guinea pigs. With two tuberculous rabbits, it was determined that the protein from the Strain H-37 gave a rise in temperature in small doses. Rabbit R 1366 was given 1 mg. of the Protein MA-100 10 weeks after inoculation with 0.5 mg. bovine Strain B-1; the temperature fell 0.5° and then rose 2° ; 2 days later the reaction to 0.001 mg. of the same protein was similar but with a rise of only 1.2° . Rabbit R 1367, inoculated with tuberculosis at the same time as R 1366, was given 0.01 mg. of the same protein in 10 weeks and showed a fall of 1.2° and a subsequent rise of 2.1° ; while 2 days later the rise in temperature after the injection of 0.0001 mg. was of the same magnitude, 2° . After all four injections in these two animals, the leucocytes rose and the lymphocytes and monocytes fell. Considering the difference in size of rabbit and guinea pig, the tuberculous rabbit inoculated with the bovine strain of organisms proved to be quite sensitive to the protein from the human strain of tubercle bacillus.

TABLE V
Effects of Distilled Water and Salt Solution in Tuberculous Guinea Pigs

Animal number	Date of infection with tubercle bacilli Hr 1,000,000 organisms	Type of fluid injected (1 cc.)	Date of injection of fluid	Route of injection	Effect on temperature	Blood count before infection. White blood cells per c. mm.			Blood count before injection of fluid. White blood cells per c. mm.			Maximum change in cells following injection of fluid. White blood cells per c. mm.			Result
						PMN	Ly	Mono	PMN	Ly	Mono	PMN	Ly	Mono	
30 R 1342	2/13/30	Distilled H ₂ O	3/24/30	i.p.	Irregular -1.9° +1.7°	5883	3330	1554	10089	5564	1770	—	—	—	Survival
14 R 1326	2/13/30	Distilled H ₂ O	6/ 5/30	i.p.	Negative +0.8°	9638	13806	2084	11712	4224	2880	14980	2041	1365	"
20 R 1332	2/13/30	Distilled H ₂ O	6/ 5/30	i.p.	Negative -0.7° +0.4°	1026	3564	810	5746	1774	929	7169	1422	1148	"
30 R 1342	2/13/30	Stock salt solution	4/24/30	i.p.	Irregular ±1.0°	5883	3330	1554	4260	6390	1988	15870	2760	1525	"
31 R 1343	2/13/30	Fresh salt solution	4/24/30	i.p.	Negative	6732	2856	408	6840	3720	1320	11160	1878	552	"
57 R 1425	Normal	Distilled H ₂ O	6/ 6/30	i.p.	Negative	3504	1679	1971	—	—	—	—	—	—	"

In two other tuberculous rabbits, R 1364 and R 1362, a comparison was made between the reaction of a polysaccharide isolated by Anderson from the bovine organism, analogous to the A-8 from the human strain and the polysaccharide isolated by Heidelberger from the human strain. In both instances the reaction was practically identical and is shown in Chart 7 for R 1364. A more marked rise in temperature following the Heidelberger polysaccharide was shown by another rabbit, R 1365, Chart 8. The chart illustrates very well the delayed return of the temperature to the original already noted.

Controls

The polysaccharides, the Protein 304, and the alkali-soluble protein were all injected into the guinea pig in 1 cc. water freshly distilled from glass. In the rabbits 2 cc. of fluid were used. The Protein MA-100 was received from the H. K. Mulford Company in sealed ampules in sterile salt solution. It was thus necessary to study the effects of these diluents. The freshly distilled water or salt was boiled and used as soon as sufficiently cooled. Neither the water nor the freshly made salt solution gave any rise in temperature. The curves of temperature for distilled water and for a stock salt solution are shown in Chart 4, and the records are given in Table V. In general both the distilled water and the salt solution did not give a positive curve in temperature. The distilled water was either negative or showed a slight fall as on Chart 4. The stock salt solution, which had been kept in sterile flasks gave a slightly more irregular curve, Chart 4, than the freshly prepared solution.

In these experiments it has been found that control temperatures at hourly intervals should be taken for 2 or 3 days preceding each experiment and after each experiment until the temperature reaches the original level. This may take 4 or 5 days.

Concerning these control temperature reactions, two factors must be considered; first, the level at which the temperature runs, and second, the steadiness of the reaction during the day. Our charts show differences in level of as much as 2°. This difference in the animal is probably related to differences in atmospheric temperature, which, of course, could only be tested when the animals were not in artificially heated rooms. The irregular temperature reactions are more difficult to evaluate because more factors may be involved. It is probable that variations in humidity can affect the temperature of the animal, but abnormal conditions, such as accidental infections must also be taken into consideration.

proteins from other sources. Arneth (23) then demonstrated that 8 hours after the injection of peptone intravenously in rabbits, at the height of the leucocytosis, there were 3 per cent myelocytes in the blood stream, showing a replacement of leucocytes from the bone marrow. In 1923 Hussey (24) discovered that the same phenomenon occurred after the injection of a considerable series of salts, sodium chloride, sodium carbonate, potassium phosphate, lithium nitrate, and sodium sulfate, and stressed the fall in mononuclear cells, finding that it was about 70 per cent in 3 hours. More recently Beard and Beard (25) have followed the blood cells in eight rabbits every 10 minutes for 5 hours after the intravenous injection of from 10 to 15 cc. of salt solution varying from 1 to 2.5 per cent. By making the counts at such frequent intervals they demonstrated that all three groups of the white cells, leucocytes, lymphocytes, and monocytes, fall immediately after the injection and that the maximum leucopenia is reached in about an hour. At this time the neutrophilic leucocytes start to rise, while the lymphocytes and monocytes continue to fall. The monocytes then begin to rise but in their experiments did not reach their original level in 5 hours, while the lymphocytes were lower by approximately 2000 cells at the end of the experiment.

This type of reaction is well shown for the tuberculous guinea pig, Chart 4, R 1342. By taking the count 2 hours after injection, the fall in the leucocytes was missed but the subsequent rise is clear. It will be noted that before each injection shown in this chart, the lymphocytes were high and showed a marked fall with the lowest level, with one exception, at the end of 7 hours. They had recovered in varying degrees by the next morning, but only in one instance did they exceed the level of the time before the injection. The monocytes varied somewhat in reaction in these tuberculous guinea pigs, but as shown in Chart 4, after a fall during the first 2 hours, they showed a tendency toward a temporary recovery in 5 hours with subsequent fall at the seventh hour.

As is shown in Table V the effect of the injection of distilled water on the blood cells may be slight as in Guinea pig R 1326, or negative as in R 1332; while the injection of salt solution is followed by the characteristic changes already described.

It is clear that the injection of proteins, salts and sugars has constant effects on the blood cells at quite specific time intervals. There is an immediate leucopenia followed by a leucocytosis, in which the three strains of circulating white cells fall at different rates and return at different times. The leucocytes start to return first; then the monocytes, and finally the lymphocytes. This reaction is elicited in the tuberculous animal by all the proteins tested; it also follows the injection of polysaccharide in concentrations containing an amount of nitrogen too small to cause a temperature reaction. The rise

Effects of the Injection of the Tuberculo-Proteins and Polysaccharides on the Blood Cells

In this series of experiments only immediate effects of the injection have been considered. The blood counts for tuberculous guinea pigs after protein and polysaccharide are shown in Tables II and III.

The total numbers of neutrophilic leucocytes, lymphocytes, and monocytes are given before the infection with tubercle bacilli, in the first column; in the second column are the corresponding figures for the count taken just before the injection of either protein or sugar; while in the third are recorded the greatest change in these three strains. This third column therefore does not represent a single count as was true for the first two columns. After the injection of the protein there was a rise in leucocytes in every guinea pig except the second animal in Table II, R 1321, which died in 16 hours; this was the only one of the fatal cases in which counts were made. In every animal there was a fall in lymphocytes, which for the most part showed little or no tendency to recover; the monocytes have also fallen in every instance but have shown a tendency toward a subsequent rise before the end of the 7 hours of the experiment. The average fall in lymphocytes in tuberculous guinea pigs after the protein, computing from the percentages rather than from the total numbers, was 63 per cent.

After the injection of the polysaccharides the total numbers of lymphocytes fell in every instance but one; this case was the last experiment in Table III, R 1319, shown in Chart 5, after the injection of 0.01 mg. of the Heidelberger polysaccharide, in which the lymphocytes were very low before the injection. In this animal, the total numbers of lymphocytes rose slightly but the percentages fell. In two instances, the percentage of lymphocytes rose while the total numbers fell; (R 1324 after A-8 on 3/13/30, and R 1327 after the bovine polysaccharide on 3/13/30). Including these percentages in the average, the lymphocytes fell 45 per cent after the polysaccharides, as is shown in Table III. Six normal guinea pigs showed a fall in lymphocytes of 55 per cent in 7 hours after tuberculo-protein, and eight showed a fall of 65 per cent after tuberculo-polysaccharide.

Non-Specific Protein Fever

It has long been known that the injection of proteins causes fever and a leucocytosis.

In 1890 Buchner (19) showed that bacterial proteins injected subcutaneously gave a local reaction of aseptic pus. The mechanism of this phenomenon was then studied by Roemer (20, 21) who found that an intravenous injection of bacterial protein gave a leucocytosis which was maximum in 8 hours. Goldscheider and Jacobs (22) then found that leucopenia preceded the leucocytosis using an extensive series of stimuli, organ extracts, bacterial proteins as well as

19. Buchner, H., *Berl. Klin. Wochenschr.*, 1890, 27, 1084.
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in leucocytes in tuberculous animals is not consistently greater than is recorded in the literature, though several counts of over 20,000 pseudo-eosinophilic leucocytes are shown in Tables II and III. The fall in lymphocytes of 65 per cent corresponds with the 70 per cent for all mononuclear cells recorded by Hussey. The question suggests itself of whether the tuberculo-protein and the polysaccharides may not affect the proportion of monocytes to lymphocytes and so be concerned with the cellular factors of resistance to infection.

SUMMARY

The temperature reaction in tuberculous and normal guinea pigs and rabbits is elicited by the tuberculo-protein and probably not at all by the polysaccharides. The polysaccharides may have some killing power under certain conditions, but this is not as consistently related to dosage as in the case of the proteins. Both proteins and polysaccharides cause a change in the white blood cells when introduced by any route.

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were placed on a diet consisting of a daily ration of approximately 250 gm. of cabbage and a weekly ration of approximately 20 gm. of hay and 50 gm. of oats. No water was given the animals to drink and they were maintained under standard laboratory conditions which have been fully described in a previous communication (4). The thyroid gland weights were obtained at the termination of the period of observation.

In the present analysis, the animals of this series were classified in two groups, namely, those entering the laboratory after September 15 and those entering after April 15. They were subdivided into groups according to the time period during which the cabbage diet had been fed. The results have been summarized in Table I.

TABLE I

Seasonal Variations in the Goiter-Producing Power of Cabbage (Baltimore), 1927-1929 Inclusive

Days on cabbage diet	Entering laboratory autumn and winter beginning Sept. 15		Entering laboratory spring and summer beginning Apr. 15	
	Number of rabbits	Average weight thyroid gland in gm.	Number of rabbits	Average weight thyroid gland in gm.
1-30	8	0.25	6	Less than 0.1
31-60	18	0.49	13	0.38
61-90	10	0.93	17	0.24
91-120	33	1.08	24	0.75
121-150	24	1.16	15	0.77
151-180	18	1.37	23	1.17
181-210	20	1.73	18	0.96

From Table I it can be seen that, in each group, the average thyroid gland weight of animals entering the laboratory during the autumn and winter months and kept on the cabbage diet was nearly twice as great as in the corresponding group which entered during the spring and summer months. This table suggests that the average goitrogenic power of cabbage was nearly twice as great during the 6 months period beginning September 15 as during the remaining months of the year. During the winter months the cabbage was derived largely from Maryland, Delaware and New York State and consisted of so-called "winter cabbage," *i.e.*, varieties which grow slowly and are allowed to

THE OCCURRENCE OF SEASONAL VARIATIONS IN THE GOITER OF RABBITS PRODUCED BY FEEDING CABBAGE

By BRUCE WEBSTER,* M.D., DAVID MARINE, M.D., AND ANNA CIPRA

(From the Department of Medicine of the Johns Hopkins University School of Medicine, Baltimore, and the Laboratory Division, Montefiore Hospital, New York)

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During observations by Chesney, Webster and Clawson (1, 2, 3, 4) in 1927, 1928 and 1929 on the production of goiter in rabbits by the feeding of cabbage, it was suspected that in the winter months (October to March) goiter was more easily produced than during the summer (April to September). It was further observed that there appeared to be a tendency toward involution of the thyroid in rabbits with very large goiters during the summer. Thus, individual animals kept in the laboratory for a year or more developed large goiters during the winter season which receded or involuted, to some extent, during the summer. No definite attempt was made at that time to demonstrate seasonal variation. In repeating the work of Chesney, Webster and Clawson on goiter produced by feeding cabbage, Marine, Baumann and Cipra (5) noted furthermore that "summer cabbage" was practically non-goitrogenic.

The present paper deals with an analysis of the data on which the earlier impressions of seasonal variation were founded, together with further experiments designed to demonstrate more systematically this phenomenon.

Observations at the Johns Hopkins Hospital.—An analysis was made of the thyroid gland weights of 247 rabbits fed on a cabbage diet and used for stock and control purposes in the department of medicine of the Johns Hopkins Medical School during the years 1927 to 1929. These animals were obtained from dealers in Maryland and Pennsylvania and in no instance was the thyroid gland clinically palpable upon admission to the laboratory. Immediately upon arrival the rabbits

* Fellow in Medicine of the National Research Council.

diet and under the standard conditions summarized above. Table II summarizes these weights. Although the series is not large enough to justify definite conclusions, it would appear that in 1927, the year in which goiter was first observed among the rabbit colony at the Johns Hopkins Hospital, the cabbage was higher in goitrogenic activity than in 1928 or 1929.

Data from Montefiore Hospital.—Marine, Baumann and Cipra in repeating the experiments of Chesney, Webster and Clawson in the spring of 1928, used the following diet:

1. Fresh cabbage—60 cal. per kilogram daily (the food value of cabbage figured as 3.1 gm. being equivalent to 1 cal.).
2. Whole oats—35 gm. weekly.
3. Alfalfa hay—20 gm. weekly.

All the rabbits used were reared in the laboratory from a single strain (Belgian). For the past 10 years this rabbit colony has been maintained on the following stock diet: Alfalfa hay—20 to 30 gm., oats—35 gm. and fresh water daily. Greens twice weekly.

The data of representative experiments are given in Table III.

In Experiment 1, imported raw Holland winter cabbage was fed from March 15 to June 5, 1928—81 days. At the end of this period the thyroids were readily palpable clinically and at operation they were from 2 to 2½ times the normal size, dark red in color and very vascular. On June 12 the 4 rabbits of Experiment 1 without iodization were started on whole summer cabbage (from New Jersey) together with 8 new rabbits as Experiment 2a. All 12 of these rabbits were fed for the following 55 days and on direct surgical examination, 11 of them were found to have thyroids only slightly enlarged and slightly more hyperemic than normal. Even the 4 that had large vascular goiters at the beginning of this experiment had regressed markedly as had been observed in Baltimore.

Experiment 4 was started October 8, 1928, in which 4 rabbits were fed for the next 56 days with whole winter cabbage from northern New York. At the end of this period all 4 had thyroids readily palpable clinically and on direct surgical examination were from 3 to 4 times normal size and very hyperemic.

Experiment 5 was started November 20, 1928, in which the 8 rabbits were fed for the following 28 days with the same northern New York winter cabbage. All the animals of this experiment had palpable thyroids on the 21st day and on direct examination they were about twice normal size, very vascular and dark red.

On April 17, 1929, experiment 41 was started, 2 rabbits being fed on imported winter Holland cabbage and 2 on Carolina summer cabbage. These were examined surgically after 22 days and those on the Holland winter cabbage had

mature fully before being harvested and placed in cold storage. During the spring and summer the cabbage used was of the early varieties. In the spring this was obtained from Florida and the Carolinas. As the season progressed, the source moved northward. This cabbage was of the rapidly growing, loose, immature "summer" varieties.

It was further evident that lots of cabbage from the same geographic location varied in their goiter-producing power from year to year,

TABLE II
Yearly Variation in Goiter-Producing Power of Cabbage, 1927-1929

Days on cabbage diet	1929				1928				1927			
	Autumn		Spring		Autumn		Spring		Autumn		Spring	
	No. rabbits	Average thyroid weight in grams	No. rabbits	Average thyroid weight in grams	No. rabbits	Average thyroid weight in grams	No. rabbits	Average thyroid weight in grams	No. rabbits	Average thyroid weight in grams	No. rabbits	Average thyroid weight in grams
1-30	4	0.15	2	0.10	—	—	2	0.10	4	0.35	2	0.10
31-60	5	0.47	4	0.47	6	0.40	5	0.35	7	0.60	4	0.33
61-90	5	0.49	3	0.10	3	1.60	8	0.33	2	0.70	6	0.28
91-120	8	0.55	10	1.21	12	1.49	7	0.40	13	1.20	7	0.63
121-150	10	1.17	4	0.66	8	1.20	6	0.90	6	1.10	5	0.75
151-180	6	1.10	12	1.30	7	1.30	5	1.18	5	1.70	6	1.05
181-210	6	1.40	7	0.95	7	1.10	6	1.10	7	2.70	5	0.94
Average. . .		0.76		0.68		0.80		0.61		1.19		0.58

apart from the seasonal variation described above. For example, it was observed that cabbage harvested in northern New York State during late October in 1928 was fully twice as active in goitrogenic power as the same variety of cabbage from the same locality in October, 1929, when tested in a standard way. Although no systematic effort had been made to compare this yearly variation during the early years of the work, nevertheless, data in the form of thyroid gland weights of control animals on the cabbage diet were available since 1927. These rabbits were maintained on the standard cabbage

TABLE III
Seasonal Variations in the Goiter-Producing Power of Cabbage

Experiment No. and kind of cabbage fed	Rabbit No.	Sex and weight gm.	Daily amount cabbage fed. gm. <i>60 cal. per kg.</i>	Thyroid weight gm.	Feeding period	Condition of thyroid found at operation
1 Winter cabbage (Holland)	618	M 3335	600		1928	2 times normal size, very vasc.
	619	F 2755	500		3/15 to 6/5	2½ " " " "
	620	F 3510	650		"	2½ " " " "
	621	F 2878	550		"	2½ " " " "
2a Whole summer cab- bage	618	M 3436	630		6/12 to 8/7	2 times normal size, very vasc.
	619	F 2610	490		"	Not enlarged, slightly hyperemic
	620	F 3103	521		"	" " " "
	621	F 2631	490		"	" " " "
	627	F 3050	568		"	2 times normal size, very vasc.
	628	F 2838	510		"	Slightly enlarged, mod. vasc.
	629	F 2086	388		"	" " " "
	630	F 3081	574		"	" " " "
	631	F 2685	500		"	Normal size, not vasc.
	632	F 2804	522		"	" " " "
	633	F 2238	419		"	Slightly enlarged, slightly vasc.
	634	F 3039	565		"	" " " "
4 Whole winter cab- bage	653	F 2644	506		10/8 to 12/4	3 times normal size, very vasc.
	638	F 3339	621	1.40	"	4 " " " "
	639	F 2911	543	1.02	"	3 " " " "
	640	M 2765	515	1.50	"	3 " " " "

thyroids more than 3 times normal size and very hyperemic, while in those on summer cabbage, one was slightly enlarged and the other about normal size and slightly hyperemic. Another experiment, No. 55, was started August 7, 1929, and the 4 rabbits were fed with Long Island and New Jersey summer cabbage. On direct examination at the end of 41 days the thyroids were possibly slightly enlarged and slightly hyperemic, *i.e.*, the cabbage used had little or no goitrogenic activity.

These data include the study of the goiter-producing power of cabbage during 2 winter periods and 2 summer periods. The winter periods include data on both imported Holland and New York State cabbage, while in the summer periods only cabbage grown along the Atlantic seaboard was used. The data obtained independently in Baltimore and in New York therefore are in agreement and clearly show that there is a tremendous seasonal variation in the capacity of cabbage to produce goiter in rabbits. Cabbage marketed in the fall of 1927 and 1928, whether grown in Holland or in the United States, was extremely effective, while cabbage grown along the Atlantic seaboard during the spring and summer of these years was almost ineffective.

In the light of these findings we began in the autumn of 1929 a systematic survey in an effort to demonstrate more completely the seasonal variation in the goitrogenic power of cabbage and to establish a standard with which surveys in subsequent years could be compared.

Arrangements were made so that lots of northern New York State cabbage, from the vicinity of Rochester and Syracuse, were procured at weekly intervals from September 12 to December 15. The goiter-producing power of each lot of this cabbage was tested by feeding for a period of approximately 3 weeks to rabbits with normal thyroid glands. At the same time, the iodine absorbing power of each lot was determined at frequent intervals by iodometric titration. Table IV shows the results of this survey.

It will be noted that the goiter-producing power was very low until November 8, 1929. The batch received on this date showed an abrupt increase in goiter-producing power. Although batches were tested at weekly intervals until January 4, 1930, the goitrogenic activity remained nearly constant. A similar increase in the goitrogenic activity had been noted early in November, 1928. However, one very striking difference was noted, namely, that all the winter cabbage,

whether domestic or imported Holland, procured in the fall and winter of 1928-29, was powerfully goitrogenic (producing palpable goiters in from 7 to 10 days), whereas the cabbage obtained during the fall and winter of 1929-30 was, at its best, only moderately goitrogenic, that is, the thyroids were not palpably enlarged after 21 days feeding.

TABLE IV

Seasonal Variation in Goitrogenic Activity of Cabbage, Autumn 1929

Cabbage lot No.	Source of cabbage	Experiment begun	No. rabbits used	Duration of exp. in days	Goitrogenic activity	No. cc. N/100 I absorbed by 10 gm. cabbage
<i>1929</i>						
1	Onandaga Co., N. Y.	Sept. 12	8	19	Low	2.8
2	"	" 20	4	18	Very low	4.0
3	"	" 28	4	18	Moderately low	3.7
4	Westchester Co., N. Y.	Oct. 9	4	16	Low	2.4
5	Monroe Co., N. Y.	" 12	6	18	"	3.9
6	Genesee Co., N. Y.	" 19	6	18	Very low	3.5
7	Cortland Co., N. Y.	" 26	4	17	" "	3.9
8	Madison Co., N. Y.	" 31	4	19	Low	3.9
9	Genesee Co., N. Y.	Nov. 8	4	18	Moderate	3.0
10	Monroe Co., N. Y.	" 15	6	20	Moderately marked	2.7
11	"	" 22	6	15	" "	2.4
12	Dutchess Co., N. Y.	" 27	4	21	Moderate	3.0
13	Monroe Co., N. Y.	" 29	4	19	Moderately marked	2.8
15	"	Dec. 5	10	17	" "	2.5
16	"	" 12	4	18	" "	Red cabbage
17	"	" 12	4	18	" "	2.8
19	Niagara Co., N. Y.	" 19	4	22	" "	2.6
20	Monroe Co., N. Y.	" 20	4	18	" "	Red cabbage
<i>1930</i>						
21	"	Jan. 4	6	18	" "	2.9

In view of the demonstration of an apparently antigoitrogenic substance in plants that is roughly measurable by iodometric titration (6), we made from 6 to 11 iodometric titrations on each batch of cabbage received and the averages of these titrations are given in Table IV. In general they show what had been previously demon-

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5	Whole winter cabbage	654 661 655 656 657 658 659 660	M 1636 F 2196 M 1774 M 1736 M 1550 F 2257 F 1594 F 1945	305 409 330 323 288 420 297 362	0.46 0.62	11/20 to 12/18 " " " " " " " "	2 times normal size, very vasc. 2 " 2 " 2 " 2 " 2 " 1½ " 2 " 2 "
41	Whole summer cabbage	723	M 1826	425	0.14	4/17 to 5/9 " " "	Normal size, slightly vasc. Slightly enlarged, slightly vasc. 3 times normal size, very vasc. 3 " " " " "
		738	F 1030	242			
		740	F 1110	260			
		657	M 1865	434			
55	Summer cabbage	773	M 1205	280	8/7 to 9/17 " " "	Normal size, slightly hyperemic " " Slightly enlarged, mod. " Normal size, slightly "	
		775	M 910	212			
		789	F 1135	264			
		790	F 975	227			

41

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DISCUSSION

The seasonal variations in the goiter-producing power of cabbage, and also the variations from year to year, offer extensive fields for further investigation. The existence of such variations is indicated by the data contained in Table II, which were collected in the early period of the work at the Johns Hopkins Hospital. It is further confirmed by Marine, Baumann and Cipra's observation of a marked difference in goitrogenic power of cabbage from the same locality in 1929 as compared with 1928. The evidence at hand suggests that this seasonal variation is in part dependent upon the variable amounts of a goitrogenic factor and an antigoitrogenic factor. The existence of the latter substance seems to be established. Although in all probability climate rather than soil is the influence determining the amount and proportions of these two factors, it is not possible at present to correlate the meteorological data for the years 1927, 1928 and 1929 with the variations in goitrogenic activity of cabbage in the same time period. The nature of the annual variations is still unknown. The survey made in 1929 and summarized in Table IV marks the beginning of a systematic attempt to determine the extent and nature of these variations and it is our intention to continue these observations over a period of years. At the end of a sufficient time period, comparison of these results with meteorological and other data may throw some light on the factors responsible for the variations noted.

SUMMARY

The evidence presented indicates that cabbage maturing in the spring and summer months has little goiter-producing power. Cabbage maturing in the late autumn has much greater goiter-producing power, although this shows considerable variation in different years. It has not been possible to correlate available meteorological data with these variations.

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strated—that the higher the iodine absorbing power the lower the goiter-producing power, that is, that the goitrogenic index varies inversely with the iodine absorbing power. Red cabbage from the same source had approximately the same ability to produce goiter as did the white variety. The possibility of climatic conditions influencing this seasonal variation in goitrogenic activity was considered. Accordingly, meteorological data were obtained from the United States Weather Bureaus at Syracuse and Rochester, New York. The normal mean temperature for October and November, 1929, was considerably higher than in 1928, as was the total number of hours of sunlight. On the other hand, the total precipitation was less in 1929 than in 1928. Continuous frosts occurred from October 10 in 1929, although the first killing frost was not until October 18. This was 10 days earlier than in 1928. Nothing was found in the climatic variations in the autumns of 1928 and 1929 that could be correlated with the great differences in goiter-producing power of the cabbage grown in these two years. It is hoped, however, that by collecting data in this standard way for at least 5 years some correlation will be possible.

In the autumn of 1928, it was found that cabbage imported from Holland, where the growing season is essentially long and slow, was high in goitrogenic activity. The same variety from approximately the same region was found to be only moderately active in 1929.

Although it has been apparent from the beginning that the variations in the goiter-producing power of cabbage were far beyond the range of anything which could depend on the iodine content, nevertheless determinations of the iodine content of samples of New York State and South Carolina cabbage were made.* The dried whole New York cabbage was found to contain 98 parts of iodine per billion. On this basis the fresh leaves would contain approximately 10 parts per billion. The iodine content of dried cabbage grown near Charleston, S. C., was 109 parts per billion. The goitrogenic activity of these two lots, with approximately the same iodine content, was found to differ widely. The Charleston sample had little or no goitrogenic activity, while the New York State sample, when tested under the same conditions, was moderately goitrogenic.

* We are indebted to Dr. Roe E. Remington of the South Carolina Food Research Laboratory, Charleston, for making the iodine determinations.

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quantity of SSS had to be elaborated. Since the point of maximum precipitation is difficult to judge and may not necessarily be the point of complete precipitation, the method devised consisted essentially of a rough titration of the antiserum with the SSS. In principle the method was similar to the one used by Pick (2) in the salt fractionation of proteins.

To constant amounts of serum, varying amounts of SSS (the quantities increasing by 0.02 to 0.03 mg. for each cubic centimeter of serum) are added. The mixtures are incubated in the water bath at 37°C. for 1 to 2 hours and then are put in the ice-box for 2 hours or overnight. They are then centrifuged, and 0.02 mg. or 0.03 mg. SSS per cubic centimeter of serum is added to each supernatant; the stay in water bath and ice-box incubation is repeated. The supernatants of the mixtures in which complete precipitation occurred, show no further precipitation; the mixture containing the least amount of SSS, which fails to show further precipitation is considered to contain the required quantity of SSS. The following protocol illustrates one such titration:

2 cc. serum	+ 0.6 cc. 1-5000 (SSS)	→Ppt.	- Supernat.	+ 0.2 cc. 1-5000 (SSS)	→Ppt.
" "	+ 0.8 cc. " "	→Ppt.	- " "	+ " " "	→ "
" "	+ 1.0 cc. " "	→Ppt.	- " "	+ " " "	→ "
" "	+ 1.2 cc. " "	→Ppt.	- " "	+ " " "	→No ppt.
" "	+ 1.4 cc. " "	→Ppt.	- " "	+ " " "	→ " "

From this titration it may be seen that 1.0 cc. of a 1-5000 solution of SSS (in saline) per 2 cc. of serum was insufficient, whereas 1.2 cc. sufficed for complete precipitation. For more exact determination of the required amount of SSS, tests may be performed with quantities ranging between 1.0 to 1.2 cc. of 1-5000 SSS/2 cc. serum; this yields a value of 0.1 to 0.12 mg. SSS per cubic centimeter of serum. When the supernatants are incubated with additional SSS, a slight precipitate frequently settles out after prolonged ice-box incubation (48 hours), which was not perceptible after 2 hours' ice-box incubation. Whether this additional precipitate is specific or not may be determined by the fact that specific precipitate (after pouring off the supernatant) is soluble in dilute acid whereas sedimented denatured serum protein is insoluble in dilute acid. For the experiments here recorded, the readings were made after 2 hours' ice-box incubation, since it was found that the supernatant failing to show any precipitation at that time always had some excess of SSS.

A phenomenon similar to the "Danysz effect" in the combination of toxin with antitoxin was observed in relation to the combination of SSS with homologous precipitin; more SSS was required for complete precipitation when the total quantity was added at once than when it was added in fractions of the total on successive days. For example, 0.16 mg. SSS/cc. serum was required for complete precipitation when added at one time, whereas only 0.13 mg. SSS was sufficient

ON THE PRESENCE IN ANTIPNEUMOCOCCUS SERUM OF TYPE-SPECIFIC PROTECTIVE ANTIBODY NOT NEU- TRALIZED BY HOMOLOGOUS SPECIFIC SOLUBLE SUBSTANCE*

By ALBERT B. SABIN

(From the Department of Bacteriology and Immunology, New York University and
Bellevue Hospital Medical College, New York)

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It has been generally assumed that the soluble specific carbohydrate of pneumococcus can neutralize the type-specific protective action of antipneumococcus serum. The purpose of the present communication is to present experiments which indicate that antipneumococcus serum also contains type-specific protective antibody not neutralized by the homologous soluble specific carbohydrate.

EXPERIMENTAL

The antipneumococcus horse serum used in these experiments was supplied by courtesy of Dr. Wm. H. Park. The soluble specific substance used was prepared by Dr. H. Sobotka, and on analysis has proved practically identical with that of Heidelberger, Goebel and Avery (1). The mouse protection tests were performed at the Research Laboratories of the New York City Department of Health under the direction of Miss Georgia Cooper, to whom the author is greatly indebted. The protective unit of antipneumococcus serum, to which reference is made later on, may be defined as the smallest amount of antiserum, which will protect mice, inoculated intraperitoneally with 100,000 fatal doses of a fully virulent culture, for 96 hours.

Method of Total Precipitation of Anticarbohydrate Precipitins

In all the experiments it was necessary to know the amount of soluble specific substance (SSS) required for complete precipitation of the antiserum, i.e., for the removal of all detectible anticarbohydrate precipitins. A standard simple method for determining this

* This study was aided by a grant from the Littauer Fund for Pneumonia Research in New York University.

next 24 hours had no appreciable SSS. 4½ hours after injection both rabbits were bled, and the serum was tested for SSS, precipitin, and protective antibody.

The results shown in Table I indicate that although the precipitins were neutralized beyond the limits of detection, the protective antibody was only partially neutralized, leaving approximately 50 to 60 per cent. The quantity of SSS that was lost in the urine was small compared with the amount injected, and since the precipitins were completely neutralized it may be assumed that most of it combined with the antibody. It is realized that the mouse protection test is

TABLE I

In Vivo Neutralization of Precipitin and Protective Antibody by SSS

Rabbit	Substances injected	Serum drawn 4½ hours post injection		
		SSS	Precipitin	Protective antibody
A	5 cc. serum (I) 1.0 mg. SSS I	Negative	Negative	3 units/cc.
B	5 cc. serum (I) Control	Negative	Positive 1-10	5 units/cc.
C	5 cc. serum I 1.0 mg. SSS	Negative	Negative	2 units/cc.
D	5 cc. serum I Control	Negative	Positive 1-10	4 units/cc.

not sufficiently accurate to permit of mathematical deductions as to the quantity neutralized; yet the figures are definite enough to show that there is considerable protective antibody, presumably free from demonstrable anticarbohydrate precipitins, which apparently is not neutralized *in vivo* by SSS.

Relationship between the Quantity of SSS Added and the Amounts of Precipitins and Protective Action Neutralized

The object of this experiment was to obtain additional information with regard to the relationship between the anticarbohydrate precipitins and the protective power of antipneumococcus serum from the

when 0.05 mg. was added the first time and 0.02 mg. daily to the supernatant on subsequent days.

It is realized that the method just described does not give absolute values for the amount of SSS required for complete precipitation, but the approximate values were considered sufficiently accurate for the subsequent experiments. In a quantitative study between the soluble specific substance of Type III pneumococcus and its homologous precipitin, Heidelberger and Kendall (3) showed that the entire reaction occurs in accordance with the mass law. The data obtained by my method of titration are in the zone where only SSS is detectable in the supernatant.

In Vivo Neutralization of Precipitin and Protective Antibody by SSS

Felton and Bailey (4) have assumed that SSS neutralizes the "protective bodies" of antipneumococcus serum *in vivo*, although they did not demonstrate complete inhibition of the protective action.

To determine the *in vivo* neutralization of protective antibody by SSS it was deemed advisable to combine the SSS and antiserum in one animal (rabbit), and then test the serum of that animal for protection in the mouse, instead of injecting the antiserum, SSS, and organisms in the same mouse at one time. Since it has been demonstrated that small amounts of SSS are capable of annulling the inhibitory action of the sera of naturally resistant animals on the growth of pneumococci in serum-leucocyte mixtures (5), and also that SSS can lower the resistance of mice to partially attenuated pneumococci (4a), it was intended, by this modification, to eliminate any possible effect that SSS may exert on factors involved in protection other than the specific antibody in the serum. The following is a summary of an experiment.

Two rabbits of nearly equal weight were injected each with 5 cc. of Serum 121 (Type I) intravenously; this serum had about 1000 protective units/cc. and required 0.16 mg. SSS/cc. for complete precipitation or 0.8 mg. SSS for 5 cc. serum. Rabbit A (1700 gm.) received an intravenous injection of 1.0 mg. Type I SSS (0.2 mg. excess), 5 minutes after the administration of the serum. Rabbit B (1820 gm.) received nothing outside of the 5 cc. of serum. Rabbit A voided 15 cc. of urine 10 minutes after the injection of SSS. This urine gave a positive test for SSS in a dilution of 1-4; the subsequent sample of urine voided within the

Non-Specific Adsorption of Protective Antibody by Specific Precipitate

It has been demonstrated that when a serum, containing precipitin, is precipitated with homologous precipitinogen certain other antibodies contained in it may be carried down in the precipitate. Antipneumococcus horse serum contains both so-called anti-"protein" and anti-carbohydrate precipitins; removal of either one by its homologous precipitinogen has not resulted in an appreciable decrease of the other. The results of the preceding experiment, however, suggest that protective substance may be adsorbed non-specifically by specific precipitate; to determine this possibility the following test was performed.

To 5 cc. of Type II antipneumococcus serum (101), 5 cc. of Type I serum (121) were added. The Type II serum (101) required 0.12 mg. SSS/cc. for complete precipitation, so that 0.6 cc. of a 1-1000 solution of SSS (II) was added to the mixture. After the usual water bath and ice-box incubation the precipitate was centrifuged and the supernatant tested for Type I precipitins and protective antibody. It was found that whereas the Type I precipitins were not appreciably affected (the amount of SSS I required for complete precipitation of the supernatant was the same as that for the original serum), the protective action was diminished from 1000 units/cc. of Type I serum to 400-600 units/cc. Following the precipitation of a polyvalent (Types I and II) antipneumococcus serum with Type I SSS, its protective action against Type II pneumococcus was reduced from 1000 units/cc. to 600 units/cc., whereas the Type II anticarbohydrate precipitins were not diminished as judged from the amount of Type II SSS required for complete precipitation.

Protection by Specific Precipitates

In 1915, Gay and Chickering (6) observed that the precipitates, obtained by the interaction of water-soluble extracts of pneumococcus with homologous immune serum, protected mice against infection almost as well as the original serum. They interpreted this phenomenon as meaning that the precipitate was dissociated, and the liberated antibody was free to act. Felton and Bailey (3) concluded that the specific precipitates obtained with Type II SSS have no protective capacity. It is possible, however, from a study of their protocols to reach an opposite conclusion, in which case additional evidence is given for the view that the precipitate formed by SSS and immune

manner in which different quantities of SSS would neutralize their respective activity in the serum.

The serum used in this experiment was 121 (Type I); it contained 1000 protective units per cubic centimeter and required 0.16 mg. SSS/cc. for complete precipitation. To three 10 cc. samples of the serum, 1.6 mg., 0.8 mg., and 0.4 mg., SSS respectively were added, *i.e.*, one portion received the full amount of SSS required for complete precipitation, another had one-half and the third portion one-fourth that amount. The tubes were incubated in the water at 37°C. for 2 hours, and in the ice-box overnight. The precipitates obtained with 1.6 mg. SSS and 0.8 mg. appeared equally voluminous, whereas the volume of the precipitate with 0.4 mg. was somewhat smaller. After centrifugation, the supernatants were tested for protective antibody and remaining precipitin. This experiment was repeated with another Type I serum (607).

TABLE II

Removal of Anticarbhydrate Precipitin and Protective Antibody by Varying Amounts of SSS

Protective units/cc. serum	Amount of serum	Amount of SSS I added	Protective units/cc. of supernatant	Amount of SSS for complete precipita- tion of supernatant
	cc.	mg.		mg.
121, I	10	1.6	100	0
1000 units	10	0.8	300	0.6-0.8
	10	0.4	100	1.0-1.2
607, I	10	1.5	200	0
1000 units	10	0.75	500, <1000	0.6-0.7
	10	0.32	200, <500	1.0-1.2

The data presented in Table II indicate (1) a lack of proportionality between the quantity of SSS used for precipitation and the amount of protective action left in the supernatant, and (2) that complete neutralization of the anticarbhydrate precipitins does not annul all of the protective activity of the serum. Quantitatively the data shown in Table II appear paradoxical. Although the apparent lack of proportionality between the quantity of SSS added and the amount of protective antibody neutralized might be accounted for by the possible non-specific adsorption of protective substance on the SSS-precipitin complex, it seems unwise to express a definite opinion.

stance by non-specific adsorption, the results of Gay and Chickering and those of Felton and Bailey on the protective capacity of specific precipitates may be accounted for partially on that basis, instead of entirely on the dissociation of specifically combined substances. In our own experiments the protection tests with specific precipitates were usually not so good as obtained by Gay and Chickering or as shown in some of the data of Felton and Bailey. A specific precipitate obtained with the full amount of SSS required for complete precipitation of the anticarbohydrate precipitins, always showed considerable protective action but never as much as that removed by the precipitate from the serum. The protection test performed at first with a suspension of the specific precipitate in a 1 per cent NaCl solution was later discontinued since accurate dilutions of a suspension of particles of varying size was impossible and the results were markedly irregular. The specific precipitate, however, is soluble in dilute acid, and more regular protection tests were obtained when the dilutions were made from a solution of the precipitate in $N/1000$ HCl; solutions stronger than $N/100$ HCl have a deleterious effect on the antibody, particularly when a day or more intervenes before the test is performed.

The following protocol shows the data of a characteristic experiment with Type I serum:

- (a) Original serum..... 1000 protective units/cc.
- (b) Supernatant from specific precipitate with full amount of SSS for complete ppt..... 100 units/cc.
- (c) Specific precipitate from 10 cc. serum dissolved in 10 cc. $N/1000$ HCl
..... 200 units/cc.
- (d) Specific precipitate from 10 cc. serum (with $\frac{1}{2}$ amount of SSS required for complete precipitate) dissolved in 10 cc. $N/1000$ HCl.. 200 units/cc.

The sum of the protective action of the supernatant with that of the specific precipitate represents only about 30 per cent of the total; in other experiments this sum has been found to vary between 20 to 50 per cent of the total. Whether or not the remaining protective action corresponds to that removed as a result of the neutralization of the anticarbohydrate precipitins has not been determined as yet.

Felton and Bailey (4b) showed that solutions of the specific precipi-

serum can still protect against infection with the homologous type of pneumococcus.

The data, bearing on this point, in Table III of Paper I (Felton and Bailey) are as follows:

Precipitate with 2.5	mg. SSS....	protected almost as well as control serum.
" " 0.25	" SSS....	had no protection.
" " 0.025	" SSS....	protected as well as the control serum.

Assuming that 0.25 mg. SSS is the optimum amount for complete precipitation, the precipitate produced with 2.5 mg. SSS, probably smaller on account of a slight zone phenomenon does not carry down with it all of the SSS; the specific precipitate has primarily only the combined antigen, leaving the rest in the supernatant, therefore, the precipitate with 2.5 mg. is essentially the same as the one with 0.25 mg. SSS and should not protect. Similarly, the precipitate with 0.025 mg. SSS although smaller in amount than that obtained 0.25 mg., still has both the antigen and the antibody (the excess of antibody remaining in the supernatant); yet it protects as well as the control serum.

Even if the assumption made by Huntoon (7), that an insufficient amount of antigen combines with antibody in two ways (1) a strong union, *i.e.*, specific neutralization, (2) a weak union corresponding to the adsorption of heterologous antibody by a precipitate, be applicable in this case, the results of the above experiment are still unexplained. Since in certain of the experiments, suspensions of specific precipitate had some protective action Felton and Bailey have assumed that the specific precipitate acts "only to a slight degree as a protective agent," and offer the same explanation as Gay and Chickerling, namely, that it is "due in part to dissociation before the substance is injected into the mouse, and also to dissociation within the animal." This explanation does not conform to the contention (Felton and Bailey in Paper I) that SSS neutralizes the protective substance *in vivo*. For even if extensive *in vitro* dissociation of antigen-antibody complex were possible, as long as the two reagents are not separated, their injection into the body should cause their re-union, if *in vivo* neutralization occurs; for extensive dissociation cannot be assumed to occur in the same medium where almost complete neutralization should occur.

If, as was indicated in the foregoing experiments, the combination of SSS with homologous precipitin can carry down protective sub-

tion of it is excreted in the urine immediately, and a small amount of SSS remains in the blood, where it can be detected for approximately 10 days. It is also known that antibody is not detectible in rabbit's urine. If the acid solution of specific precipitate contained dissociated SSS-antibody complex, which failed to reunite when injected *in vivo*, SSS should be found in the urine and precipitin in the blood.

Protocols.—Specific precipitate from 20 cc. of antipneumococcus serum 607 (Type I) with 3 mg. of SSS I (amount for complete precipitation) required 12 cc. of N/40 HCl to dissolve completely; this was slowly injected into the marginal ear vein of a rabbit (2260 gm.). 4 hours after injection blood was drawn from the marginal vein. This rabbit serum had no demonstrable SSS or precipitin; it was tested for protective antibody in the range of 0.5 to 5 units per cc. and was found to contain 2 protective units/cc. The total amount of urine voided within the next 16 hours was 10 cc.; it had no demonstrable SSS. In other similar experiments with Type I specific precipitates faint traces of SSS were occasionally detectible in the urine for a period of 24 to 48 hours after injection, but never any SSS or precipitin in the serum although it always had protective action.

When the original urine failed to give a test for SSS it was concentrated as follows: 10 cc. or more of urine was dialyzed for 24 hours in running water. The contents of the bag were then heated with a few drops of dilute acetic acid until it evaporated to 5 cc.; after filtration, 10 volumes of 95 per cent C_2H_5OH were added and the precipitate collected by centrifugation. The precipitate was dissolved in 1 cc. of 1 per cent NaCl solution and neutralized when necessary with N/50 NaOH.

These experiments show that when an acid solution of specific precipitate is injected intravenously into a rabbit, there is no indication that the SSS-antibody complex exists in a dissociated state. In two experiments, the urine had no appreciable SSS and the rabbit serum, containing no demonstrable SSS or precipitin, had 2 units of protective antibody per cubic centimeter. In the experiments where SSS did appear in the urine it was so slight in amount compared with the quantity injected, combined in the specific precipitate, that it may represent uncombined excess which was either introduced as such or resulted from slight dissociation. That appreciable dissociation did not occur is evident from the fact that at no time was anticarbohydrate precipitin demonstrable in the rabbit serum. These experiments, therefore, bring additional evidence to the view that the protective action of the specific precipitates is probably due to the liberation of non-specifically adsorbed protective antibody.

tate in certain acid and alkaline zones exhibit marked protective action; their experiment was as follows.

Some specific precipitate was dissolved in $N/20$ HCl and a number of samples were adjusted to different pH, ranging between 4.4 and 9.8. After this adjustment, any insoluble material was centrifuged out, and the supernatants were used in tests for protective antibody, precipitinogen, and agglutinin (precipitin). Abundant protection was obtained with the supernatants from the samples adjusted in the range of pH 4.4–5.3 and pH 8.4–9.8. Our own experiments indicate that the specific precipitates are practically wholly soluble in these pH ranges and almost insoluble at neutrality.

Felton and Bailey believed that their solutions contained two dissociated components (1) protective antibody, (2) SSS. Even if this assumption were true, the results of their protection tests are difficult to explain on this basis, since not having separated the two components, the injection of these solutions into the body ought to result in their reunion. But the precipitinogen and agglutinin tests with these supernatants, upon which the contention of the dissociated state of the precipitate is based, are not valid as is indicated by our experiments, which showed that precipitation obtained by the addition of homologous serum (test for precipitinogen) to solutions of specific precipitate in the pH range 4.4–5.3 and 8.4–9.8 was non-specific, since normal serum and buffer solutions of a pH about 7.0 gave the same results. The precipitation is apparently due to the shifting of the pH to the zone of insolubility of the SSS-precipitin complex by the serum or buffer solution. The addition of homologous SSS to these solutions also precipitates them non-specifically, heterologous SSS producing the same effect. It thus appears that the evidence is against supposing that the solutions represented dissociated antigen-antibody complex, so that their protective action must be accounted for on another basis.

To obtain further information on the possibility of such dissociation and to determine the extent of *in vivo* dissociation of SSS-antibody complex, advantage was taken of the fact that the rabbit kidney is virtually a selective filter with regard to SSS and antibody. It is well known that the urine of a pneumococcus-infected rabbit contains SSS; our own experiments indicated that within a short time after the intravenous injection of purified SSS into a rabbit, the greatest por-

pernatants was never neutralized by SSS. From these results, taken together with the observations of the foregoing experiments, it may be concluded that there is in antipneumococcus serum type-specific protective antibody which is not neutralized by SSS and apparently distinct from the anticarbohydrate precipitins. The amount of this protective antibody in an antiserum is difficult to estimate, but it is undoubtedly more than the residue found in the supernatant since a considerable quantity is probably adsorbed apparently non-specifically on the SSS-precipitin precipitate.

Is There Another Type-Specific Antigen in the Pneumococcus in Addition to the SSS?

The contention that there is a type-specific protective antibody, not neutralized by SSS, necessarily presupposes the existence of another antigen. Since each antibody must have its corresponding antigen, complete proof for the existence of a type-specific antibody distinct from the anticarbohydrate precipitin will not be available until a type-specific neutralizing antigen is found which is distinct from SSS. The following experiment offers an indication that such an antigen may actually exist.*

Protocols.—The serum used was Type I (607) containing 1000 protective units/cc.; the quantity of SSS required for complete precipitation was 0.15 mg./cc. 20 cc. of Serum 607 was precipitated with 3 mg. of SSS I. After the routine water bath and ice-box incubation, the supernatant obtained by centrifugation, was divided into several portions, which were treated as follows:

- (a) Supernatant + 0.075 mg. SSS/cc. (50 per cent excess of SSS).
- (b) " + 0.15 mg. SSS/cc. (100 per cent " " ").
- (c) " 4.6 cc. + 2 cc. emulsion of Type I pneumococci (heatkilled) + 1.4 cc. saline.
- (d) Supernatant 4.6 cc. + 2 cc. emulsion of Type II pneumococci (heatkilled) + 1.4 cc. saline.

The organisms used for absorption were obtained each from 200 cc. of an 18 hour broth culture (heated at 56° for 30 minutes) by centrifugation. The sedimented organisms from 200 cc. were emulsified in 4 cc. of saline.

* Since the writing of this communication, Enders, J. F., *J. Exp. Med.*, 1930, 52, 235, presented evidence "for the existence of a type-specific substance distinct from the specific carbohydrate in the autolytic products of *Pneumococcus* Type I."

Protective Action of Antipneumococcus Serum after Complete Precipitation of the Anticarbhydrate Precipitins

At the very beginning of this investigation it was observed that after complete precipitation of the anticarbhydrate precipitins from Type I antipneumococcus sera, the supernatant always had some residual protective action, the amount varying from 10 to 30 per cent of the original serum. The residual protective antibody in the supernatants of Type II sera were considerably less, but nevertheless definite.

TABLE III
Protective Action of Antipneumococcus Serum after Complete Precipitation of the Anticarbhydrate Precipitins

Serum	Type	Amount SSS required for complete ppt./cc.	Protective units in original serum	Protective units in supernatant	Protective units in supernatant + 50 per cent excess SSS	Protective units in supernatant + 100 per cent excess SSS
		mg.				
121	I	0.16	1000	100-300%	100-300%	100-300%
170	I	0.04	100	20	20	20
607	I	0.15	1000	200	200	200
Concentrated Felton	I	0.24	2000	500	500	500
101	II	0.12	300	10	10	10
40	II	0.08	500	20, <50 200, <500*	Not done	Not done

Different supernatants tested on various occasions gave values ranging between 100 to 300 protective units/cc.

* This figure represents the protection afforded by the suspension of the specific precipitate in the supernatant.

When these supernatants were tested against a heterologous type of pneumococcus, they showed no appreciable protective action. It was thus evident that the protective antibody remaining in the supernatants, which were practically free of anticarbhydrate precipitins, was type-specific. To determine the relation of this type-specific protective antibody to SSS, the supernatants were mixed with additional SSS in amounts equivalent to 50 per cent and 100 per cent of the quantity originally required for complete precipitation. As may be seen from Table III, the residual protective antibody in the su-

2. Neutralization of the protective action by SSS *in vivo* and *in vitro* (4).

3. Absorption of the protective antibody and the type-specific agglutinins with S-producing pneumococci (Type II) as well as with the filtrates of their broth cultures (11).

In the foregoing experiments it was shown that *in vivo*, the SSS of Type I pneumococcus only partially neutralizes the protective antibody, and that specific precipitates resulting from the *in vitro* union of SSS with anticarbohydrate precipitin exert a type-specific protective action. Heidelberger and Kendall (3) observed that the SSS of Type III pneumococcus can combine with its homologous antibody in varying proportions. Should the same principles apply to the combination of the SSS of other types of pneumococci with their antibodies, it would be theoretically possible for certain specific precipitates to react with SSS in the organisms, and thus exert protection; similarly the specific precipitates, having the maximum amount of SSS in combination should not be protective. However, in the foregoing experiments the precipitates obtained with the maximum amount of SSS still showed definite protection; since it was observed that these precipitates can adsorb non-specifically heterologous protective antibody, it appeared possible that their homologous protective action might similarly be due to a liberation of non-specifically adsorbed antibody.

After the total precipitation of the anticarbohydrate precipitins, Type I antipneumococcus serum retained a definite amount of its type-specific protective action, which was not neutralized by additional SSS, although it was absorbed only with the homologous virulent pneumococci. It appears possible, therefore, that this residual protection is exerted by an antibody distinct from the anticarbohydrate precipitins. The supernatants containing the residual protection had a low agglutination titer for the homologous pneumococcus, but nevertheless somewhat higher than for heterologous pneumococci; whether or not this fact bears any relationship to the protective action does not appear definite.

The portions of supernatant mixed with SSS and those with the organisms were thoroughly shaken and incubated in the water bath at 37°C. for 2 hours and then in the ice-box overnight. It is interesting to observe especially in view of the fact that the supernatant had no appreciable protection for Type II pneumococcus, that it agglutinated both the Type I and the Type II pneumococci; the agglutination titer for Type I was 1-16 and for Type II pneumococcus, 1-8. After incubation, the organisms were centrifuged and all the supernatants were tested for protection. The results of the test were as follows:

Original serum.....	1000 protective units/cc.
Supernatant.....	200 " "
Supernatant 50 per cent excess SSS.....	200 " "
" 100 per cent " ".....	200 " "
Supernatant treated with Type I pneumococci.....	less than 50 protective units/cc.
Supernatant treated with Type II pneumococci.....	200 " "

It will be seen that the residual protective antibody in the supernatant is not neutralized by SSS nor by absorption with Type II pneumococci, but is definitely absorbed with the homologous Type I pneumococci. The agglutination of the organisms which occurred in the supernatant probably did not cause non-specific adsorption, since with the heterologous pneumococci there was no diminution in the protective antibody present. It may be assumed, therefore, that this neutralization was specific, and unless SSS in the organism is capable of neutralizing antibody which SSS in solution cannot, it must be conceded that some other substance in the pneumococcus was the responsible agent.

DISCUSSION

The mechanism whereby antipneumococcus serum averts death of a pneumococcus-infected animal is not understood. The study of the substances which may neutralize this protective activity should yield valuable information on the problem of its mode of action. The carbohydrate fraction of the pneumococcus has been assumed capable of neutralizing the protective action of antipneumococcus serum on the basis of the following observations:

1. "Soluble neutralizing substances" for the homologous immune bodies (8, 9) were demonstrated in pneumococcus infected serums and exudates which are known to contain SSS (10).

SUMMARY

The mutual relationship of the anticarbohydrate precipitins and the protective action in antipneumococcus sera to the soluble specific substance was investigated. The assumption is made that there exists in antipneumococcus serum, type-specific protective antibody which is distinct from the anticarbohydrate precipitins and is not neutralized by the soluble specific substance. This assumption is based upon the following observations in experiments which were conducted primarily with Type I antipneumococcus horse serum.

1. Lack of proportionality between the quantity of SSS added and the amount of anticarbohydrate precipitin and protective action neutralized.

2. Protective capacity of specific precipitates (SSS-precipitin complex) is accounted for on the basis of a liberation of non-specifically adsorbed protective antibody.

3. SSS only partially neutralizes the protective action of antipneumococcus serum *in vivo*.

4. Type-specific protective antibody remains in antipneumococcus serum after complete precipitation of the anticarbohydrate precipitins.

5. This residual type-specific protective antibody is not neutralized by additional SSS nor by absorption with heterologous pneumococci; it is definitely absorbed by the homologous pneumococci.

I wish to express my indebtedness to Prof. Wm. H. Park for the most valuable guidance and criticism during the course of this work.

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of ratio measurements upon a single individual and gave reason to expect highly variable results. It was therefore desirable to use a

TABLE I

No.	Age	Body weight	Body surface	*Kidney weight	Dry kidney weight	Urine urea rate	Blood urea conc.	Ratio: urine rate blood conc.	Ratio per 1000 mg. kidney
	<i>days</i>	<i>gm.</i>	<i>sq. cm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg./hr.</i>	<i>mg. %</i>		
1	390	380	600	2601	504	57	123	0.465	0.179
2	390	370	590	1966	449	33	129	0.255	0.130
3	390	330	545	2132	512	40	97	0.409	0.192
4	350	322	535	1757	405	43	115	0.370	0.210
5	350	360	575	1900	444	40	120	0.337	0.178
6	350	380	600	2300	501	55	113	0.488	0.212
7	300	380	600	2086	500	45	113	0.393	0.189
8	300	350	565	1919	458	45	122	0.365	0.190
9	300	340	555	1808	415	45	125	0.359	0.198
10	253	340	555	2200	555	55	144	0.384	0.175
11	253	325	537	1911	474	38	132	0.289	0.151
12	253	340	555	1710	442	35	135	0.259	0.152
13	174	300	510	1714	461	42	114	0.368	0.214
14	174	270	475	1788	422	41	142	0.290	0.162
15	174	320	530	2440	500	49	81	0.606	0.248
16	135	290	500	1787	446	40	121	0.328	0.184
17	135	260	463	1507	391	38	114	0.333	0.221
18	135	300	510	1739	460	48	129	0.373	0.214
19	106	200	390	1221	323	41	117	0.350	0.286
20	106	210	400	1263	328	44	199	0.223	0.177
21	106	190	380	1250	309	38	144	0.264	0.211
22	74	150	320	1035	270	39	149	0.263	0.254
23	74	160	335	1173	293	17	112	0.152	0.129
24	74	160	335	1177	294	22	71	0.312	0.264
25	56	140	310	1191	299	31	142	0.222	0.185
26	56	140	310	1122	287	38	128	0.299	0.266
27	56	150	320	1124	298	23	100	0.232	0.207
28	42	106	255	942	196	27	180	0.152	0.162
29	42	112	265	893	195	33	128	0.258	0.288
30	42	112	265	902	195	21	81	0.253	0.281
31	32	66	190	632	134	15	152	0.099	0.157
32	32	64	188	601	120	12	208	0.059	0.098
33	32	56	170	542	234	20	220	0.090	0.166

* Weight of both kidneys.

considerable number of rats of widely varying size. Thirty-three male albino rats varying in weight from 56 to 380 gm. were used.

FACTORS WHICH DETERMINE RENAL WEIGHT*

**XI. RENAL FUNCTION

BY EATON M. MacKAY, M.D., AND BURRELL O. RAULSTON, M.D.

*(From the Department of Medicine, Stanford University Medical School,
San Francisco)*

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There are certain special conditions under which the rate of urea excretion is primarily controlled only by the concentration of urea in the blood (1) and the amount of functioning renal tissue (2). Under these conditions the rate of urea excretion is directly proportional to the blood urea concentration so that the ratio:

$$\frac{\text{urine urea rate}}{\text{blood urea concentration}}$$

in a given subject remains approximately constant (3). The order of magnitude of this ratio is regulated directly by the size of the kidneys and hence becomes a measure of the amount of functioning renal tissue (4). In the rabbit it has been demonstrated (2) that the ratio is directly proportional to the weight of renal tissue. It is probable that there is a similar relationship in man for in normal individuals the ratio is directly proportional to body surface (5, 6) and in so far as one may judge from the figures given by Vierordt (7) the weight of the kidneys seems to be dependent on the body surface. In the present series of studies of the factors which determine the weight of the kidneys in the albino rat the constant use of the gross anatomical weight of these organs made it desirable to determine what relation this measure of size bears to function in the normal animal.

The small size of the experimental subject precluded the repetition

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** Earlier papers of this series have appeared in *The American Journal of Physiology* and *The Journal of Nutrition*.

food for 18 hours 30 cc. per 1000 sq. cm. of body surface of an aqueous solution containing 2.5 per cent urea and 0.7 per cent sodium chloride were injected intraperitoneally. 3 hours later the bladder was emptied by pressure over the abdomen and the administration of a few whiffs of ether (9) and 15 cc. per 1000 sq. cm. body surface of an 0.8 per cent sodium chloride solution given intraperitoneally. 90 minutes later, at the middle of a 3 hour period of urine collection, a specimen of blood was taken by heart puncture with a very small hypodermic needle. The blood specimens which were drawn were from 0.5 to 2.0 cc. depending on the size of the animal. 3 hours after the urine collection was commenced the bladder was emptied again and this specimen added to the urine and washings from the collection cage. The urea in the urine and blood was determined by the urease and aeration method and the ratio:

$$\frac{\text{urea excretion per hour}}{\text{urea in 100 cc. of blood}}$$

was calculated. Some hours later the animals were killed and the kidneys at once removed and weighed (10). They were then dried at 120°C. to constant weight. We hesitate to attach much importance to these dry weights because of the inherent difficulties in completely drying any tissue without loss.

The weights of the kidneys are compared with the ratios in Table I and all of the observations are charted in Fig. 1. These results indicate that in the albino rat as in the rabbit there is an approximately direct relation between the magnitude of the ratio:

$$\frac{\text{urine urea rate}}{\text{blood urea concentration}}$$

and the weight of the kidneys. It is very probable that a better agreement would have been found had it been possible to make repeated measurements upon each individual and had the blood specimens been more adequate in volume. However, considering these unavoidable circumstances there is a remarkable constancy in the kidney weight:ratio relationship. As might be expected the variability is higher than in the case of the rabbit. The mean ratio for each gram of renal tissue is 0.198 while the average found for the rabbit was 0.176, an agreement which is as good as might be expected. That the ratio would bear a more constant relation to body surface than to other measures of body size in the rat as in the rabbit (2) and man (5, 6) would follow from the constant relation which renal weight has been shown (11) to bear to body surface in the rat.

The special conditions under which the rate of urea excretion is directly proportional to the blood urea concentration are essentially abstinence from food (8), an increased blood urea concentration and a copious diuresis. In man the latter are easily attained by having the subject drink urea solutions and water and in the rabbit these

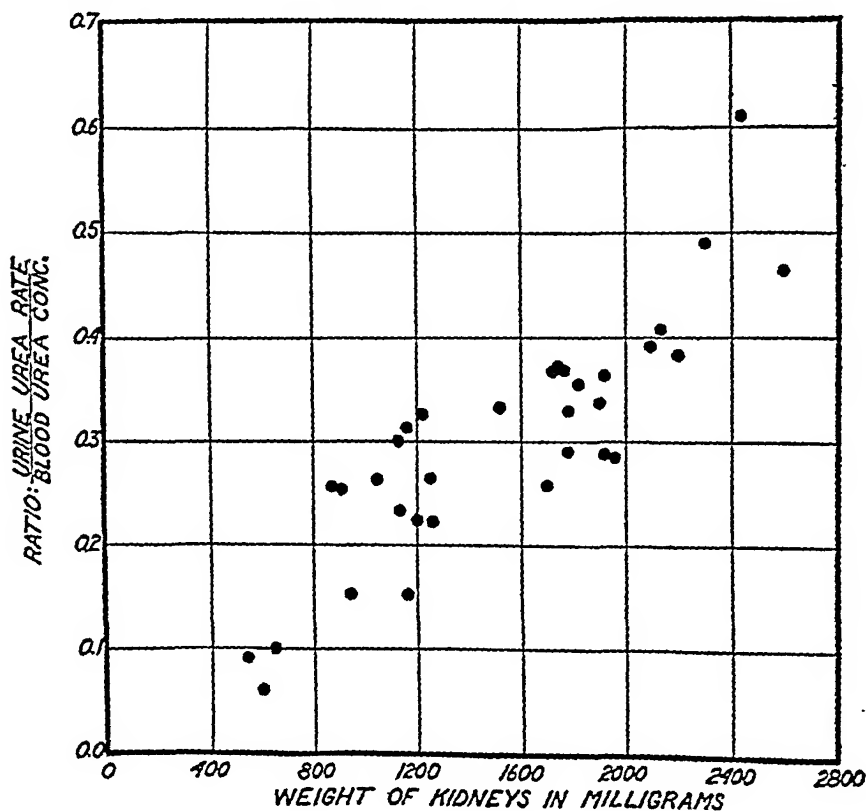


FIG. 1

fluids may be administered by stomach tube. This is practical only with very large rats and so the same conditions were brought about by the intraperitoneal injection of urea solutions.

A single ratio measurement was made on each rat. The body surface was calculated by the formula derived by Carman and Mitchell (Carman, G. G., and Mitchell, H. H., *Am. Jour. Physiol.*, 1926, 76, 380). After abstinence from



SUMMARY

The amount of functioning renal tissue as measured by the weight of the kidneys in the albino rat is directly proportional to the renal function as measured by the ratio:

$$\frac{\text{urine urea rate}}{\text{blood urea concentration}}$$

under certain special conditions.

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The experiment (6) consisted of the use of vaccine virus mixed with immune serum in such proportions that the material was without effect when inoculated intradermally in normal animals. On the other hand, in the presence of an excess of immune bodies the mixtures were ineffective, presumably because sufficient dissociation of the infectious agent did not occur.

The success achieved in the instances recorded led to the application here described of the same procedure to the virus of poliomyelitis. Highly potent virus strains were available, as were adequate stocks of human convalescent serum of proper inactivating power. Moreover, through the courtesy of Dr. W. H. Park, the globulin fraction of the serum of a horse repeatedly injected with poliomyelitis virus was also studied. The latter material had proved effective in activating poliomyelitis virus *in vitro*, both in the laboratory of the New York City Department of Health (7) and in our hands (8).

The various neutralizing sera were mixed with physiological saline suspensions of glycerolated poliomyelitic monkey spinal cord, shaken for 30 minutes at room temperature in a mechanical agitating device, allowed to stand for a similar period at the same temperature, and injected. The proportions of virus and immune serum employed were shown by experiment to be non-infectious when inoculated intracerebrally into normal monkeys. The intradermal and subcutaneous routes were chosen for the injection of the neutralized mixtures. As shown by the individual protocols, the method of testing for the presence of immune bodies in the treated animals was varied somewhat, in order to study the problem from several different aspects.

A separate experiment was carried out to determine the efficacy of the poliomyelitis virus-antiserum mixtures, intranasally instilled, to induce active immunity. When vaccine virus and immune serum were instilled into the nares of rabbits, Rhoads (6) secured a certain degree of protection against inoculations of active virus alone. Monkeys, however, receiving similarly inactivated poliomyelitis virus in considerable amounts by the same route over a period of 2 weeks, failed to show distinct and unmistakable evidence of protection against a subsequent inoculation of potent virus.

On the other hand, the intradermal and subcutaneous injections in monkeys of innocuous or noninfectious mixtures of poliomyelitis virus

IMMUNITY FOLLOWING THE INJECTION OF MONKEYS WITH MIXTURES OF POLIOMYELITIS VIRUS AND CONVALESCENT HUMAN SERUM

By C. P. RHOADS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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During the past several years the dissociation of filterable viruses from combination with specific immune sera has aroused increasing attention.

The fundamental observations of Arrhenius and Madsen (1) on the separation of diphtheria toxin from its specific antitoxin followed the classical experiments of von Behring (2), pointing out the possibility of employing such toxin-antitoxin mixtures to stimulate the production of an artificial, active immunity. Success in that field induced Besredka (3) to apply the principle to the use of sensitized bacterial vaccines. Later, Andrewes (4) investigated the possibility of separating active virus from an innocuous mixture of such a virus and specific antiserum. Both filtration through a Berkefeld candle and dilution were found to be effective in bringing about a separation. Long and Olitsky (5) brought forward additional evidence on this subject, confirming and extending the work of Andrewes, and suggesting a physico-chemical explanation of the underlying mechanism.

Following the observations concerning the dissociability of virus-immune serum mixtures, experiments were carried out in this laboratory in order to determine whether a virus can be separated from combination with the immune serum *in vivo* in such a manner as to induce a state of active immunity.

These experiments were suggested by an observation of Andrewes on a group of rabbits treated by the injection of vaccine virus in one ear vein coincidentally with an injection of immune serum by way of the other ear vein. When subsequently tested by an intradermal inoculation of active vaccine virus, part of the treated rabbits were found to be immune. Substitution of actual mixtures of virus and serum for the coincidental injection of the two factors was found by us to be equally efficacious, and the immunity so produced was of a degree enabling animals, otherwise susceptible, to resist inoculation with active virus alone.

The result of this experiment, then, can be stated as follows. Two of the animals which had received repeated intradermal inoculations of the neutralized mixtures resisted direct test and their serum neutralized the virus, whereas 2 similarly treated monkeys succumbed to inoculation and proved to have no appreciable neutralizing bodies in their serum.

Experiment II.—Four *Macacus rhesus* monkeys were given 2 sets of subcutaneous injections of equal parts of pooled human convalescent serum and glycerolated "pooled mixed" poliomyelitis virus. Each set of injections, distributed in multiple small blebs, involved a total of 30 cc. of the mixture, shaken and allowed to stand exactly as in Experiment I. The treatment was repeated after a 5 day interval, and then 28 days were allowed to elapse before blood was collected and the serum separated. This group was tested for immunity by repeated intranasal instillations of glycerolated virus suspension of the same strain as that used in immunization. Previous experimentation had shown that the instillation of 1 cc. of a 5 per cent virus suspension into each nostril, repeated daily for 3 days, was practically uniformly successful in causing infection in normal animals. This procedure was adopted as the test for immunity, since it was considered more comparable to the method of infection obtaining in man. To make the results more reliable, 2 normal animals were used as controls. 11 days after the conclusion of the series of nasal instillations, 2 treated animals and 1 control had developed typical poliomyelitis. The remaining control and the 2 surviving treated monkeys were then subjected to a repetition of the infecting procedure. The control showed characteristic symptoms 15 days after the conclusion of the first series of instillations, or on the day following the end of the second series. Bearing in mind the fact that an incubation period of 3 days or less is rare in experimental poliomyelitis, it is not unreasonable to suppose that the disease in the normal monkey resulted from the first rather than from the second set of nasal instillations.

Only the two sera of the treated monkeys which survived the foregoing procedures were tested for the presence of neutralizing antibodies. The technique was the same as described in Experiment I; 0.01 cc. of virus was mixed with 0.99 cc. of serum, incubated for 2 hours at 37°C., and kept over night in the icebox. The serum of one treated monkey neutralized, while that of the second treated monkey and the control failed to do so. It is worthy of note that the animal receiving the serum of the treated monkey showed a prolongation of the incubation period, 15 days, as compared with 8 days in the control.

In brief, then, of 4 monkeys subjected to the subcutaneous injection of 60 cc. of a mixture of equal parts of virus and immune serum, 2 survived the routine nasal instillation of virus, although 2 controls succumbed. These surviving animals also withstood a second course of nasal instillations of active virus, without evincing any symptoms of the disease. Of the sera of these 2 resistant animals, one neutralized virus, whereas the second failed to do so.

Experiment III.—Four *Macacus rhesus* monkeys were treated by 2 sets of intradermal injections of 30 cc. of a mixture of glycerolated "pooled mixed virus"

and specific antiserum induced a varying degree of protection, as brought out in the protocols to follow. The animals treated with the inactive or neutral mixtures were submitted to intracisternal, intranasal, and intracerebral inoculations of highly potent virus. The results in general were similar; one-half of the tested animals resisted an amount of potent virus sufficient to induce typical poliomyelitis in untreated controls.

As further indication that certain monkeys receiving the neutralized mixtures may attain a state of immunity to poliomyelitis, the results of tests of the specific neutralizing power of the serum of these animals may be cited. Of eight animals surviving the direct inoculation of virus, the sera of six were studied for their neutralizing capacity. Five sera neutralized or inactivated an amount of virus capable of producing typical symptoms of poliomyelitis in the control animals.

Any possibility that the outcome could be due to a passive immunity conferred by the antiserum used in the treatment was ruled out by previous experimentation. Pooled human immune serum given intravenously in amounts of 15 cc. failed to protect monkeys for more than a few days against intracerebral or intranasal virus inoculation.

PROTOCOLS

Experiment I.—*Macacus rhesus* monkeys of approximately the same size were given multiple intradermal inoculations on 20 different occasions. The material injected was a 5 per cent physiological saline suspension of glycerolated "pooled mixed virus," mixed with an equal amount of pooled human convalescent serum. Previous experiments had shown that the proportions of serum and virus employed were ample to insure neutralization. Each set of injections totalled 4 cc., and the treatments were repeated at 3 day intervals. The animals were bled 30 days after the last injection, and the serum was separated and reserved for later study. The entire group, with an untreated control monkey, was subjected to intracisternal inoculation of 2 M.L.D. of a fresh Berkefeld filtrate of the same virus strain. The control and 2 of the treated animals developed typical poliomyelitis, whereas the 2 remaining monkeys evinced no symptoms of disease. The 4 sera were mixed with fresh virus filtrate in the proportions of 0.99 cc. of serum to 0.01 cc. of filtrate, kept in the incubator for 2 hours, allowed to stand over night in the icebox, and inoculated intracerebrally into normal monkeys. The serum of the 2 animals which had resisted direct inoculation was found to be neutralizing, whereas that of the 2 which had failed to survive direct infection was lacking in this property.

allowed to elapse after the conclusion of the treatments. The animals were then bled, the serum being stored for use in subsequent experiments. Following this procedure, the entire group, with an equivalent number of untreated controls, was subjected to repeated intranasal instillations of highly potent virus alone. The control animals all developed typical experimental poliomyelitis on the seventh day following the termination of the instillations. Three of the four treated monkeys became paralyzed on the fourteenth day; the fourth showed no symptoms at any time.

It is evident from this experiment that the intranasal instillation of neutralized virus failed to give rise to demonstrable immunity under the experimental conditions observed. An adequate explanation of the superiority of the intradermal and subcutaneous routes over the intranasal can only be a matter of conjecture at this time. The fact that neutralizing substances for poliomyelitis virus are present in the nasal mucosae of normal adults may conceivably have a bearing on the matter.

DISCUSSION

The results of the experiments described in the foregoing protocols indicate that a varying degree of immunity to poliomyelitis can be induced in monkeys by the intradermal and subcutaneous injection of poliomyelitis virus neutralized by mixture with human convalescent serum. That the protection conferred by such treatments is not constant, or perhaps of high degree, is evident from the fact that only one-half of the treated animals survived the direct inoculation of virus. Furthermore, the serum of one of six survivors failed to neutralize a small quantity of poliomyelitis virus, although the remaining five effected complete inactivation. On the other hand, six of eight animals which remained well were retested by direct virus inoculation and proved resistant.

It is interesting to compare these experiments with the refractory state resulting from the subcutaneous inoculation of active virus studied by Stewart and Rhoads (9), in which it was shown that four of eight treated animals were unable to resist direct intracerebral inoculation of rather weak virus strains.

Aycock has reported that one animal in a series of twelve treated by intradermal injections of active virus developed poliomyelitis,

suspension and pooled human convalescent serum, equal parts of each being employed. This series was carried out in parallel with Experiment II, the same material being used in both sets of animals. The injections were given intradermally in this case instead of subcutaneously, as in the previous experiment. An interval of 5 days was allowed to elapse between the treatments and a rest period of 28 days between the conclusion of the treatments and the withdrawal of blood for tests of the neutralizing value. The animals received similar repeated nasal instillations of highly potent virus as in the group treated by subcutaneous injection, and the same controls were employed. Of the 4 monkeys, 2 survived 2 separate sets of intranasal instillations. The sera of these 2 resistant animals were also tested for the power to neutralize poliomyelitis virus. The same technique as previously described was employed and both sera inactivated. The control, inoculated with a similar amount of untreated virus, developed characteristic poliomyelitis.

Here once more, one-half of the treated animals proved resistant to the repeated intranasal instillation of virus, and the sera of the protected animals were shown to be effective in neutralizing virus *in vitro*.

Experiment IV.—Four *Macacus rhesus* monkeys received repeated sets of intradermal injections of glycerolated "pooled mixed virus," inactivated by admixture with 1/10th its volume of globulin fractionated horse antipoliomyelitic serum. Experimentation had demonstrated that such a preparation would render inactive a 5 per cent physiological saline suspension of glycerolated nervous tissue of the "pooled mixed virus" strain, when mixed in the proportion of 1 part of globulin to 10 parts of virus. Each treatment consisted of the injection of 15 cc. of the mixture, distributed in multiple intradermal blebs. The injections were repeated 5 times at 3 day intervals. A rest period of 1 month was allowed to intervene between the conclusion of the treatments and the tests for immunity. The first test comprised 3 sets of nasal instillations of active virus given at daily intervals. Two treated animals showed no evidence of disease following this procedure, one showed slight though definite symptoms, with excitement, ruffled fur, and mild paralysis, while the fourth as well as the control developed the clinical manifestations of poliomyelitis. After 28 days the 3 surviving animals, together with a new control, were given intracerebral inoculations of 0.01 cc. of a fresh Berkefeld filtrate of poliomyelitic nervous tissue. Although the control became prostrate on the seventh day after injection, the 3 treated monkeys remained well. In the case of the animal which had shown mild symptoms after the first test, a failure to respond to the second was ascribed to the preceding infection. That the remaining 2 monkeys developed no symptoms indicated an immunity resulting from the treatments.

Experiment V.—*Macacus rhesus* monkeys were give daily intranasal instillations of 5 per cent physiological saline suspensions of poliomyelitic nervous tissue mixed with an equal volume of pooled human convalescent serum. The amount given at each treatment was 2 cc., 1 cc. being instilled in each nostril daily for 2 weeks, making a total volume of 30 cc. of mixture. A rest period of 30 days was



an experience observed also in this laboratory. It should be noted that in the experiment reported in this communication, no animal showed symptoms of disease during treatment, a fact which is not surprising when the minimal infectivity of the inoculated material is considered. The foregoing experiments suggest that the production of immune bodies in certain animals may follow the injection of a neutralized virus ineffective in producing disease symptoms when inoculated intracerebrally in normal monkeys.

SUMMARY AND CONCLUSIONS

1. Experiments are reported on the use of poliomyelitis virus neutralized by specific antiserum as an agent to induce active immunity against the experimental disease in monkeys.
2. The results indicate that protection can be conferred upon a certain number of the treated animals.
3. The neutralized material gave rise in no instance to symptoms of disease in the treated monkeys.
4. Active poliomyelitis virus, suitably neutralized by admixture with convalescent serum, was without pathogenic effect when given by repeated nasal instillations.

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But these substances existed in the blood of normal sheep and appeared not to be increased by immunization. The method usually followed was to inject suspensions of the central nervous organs of monkeys succumbing to acute experimental poliomyelitis. One or two exceptions to the common experience were recorded. Neustädter and Banzhaf (11) (1917) believed that they had produced in a horse substances definitely inactivating to the virus when combined *in vitro*. Pettit (12) (1918) produced both in the horse and the sheep, neutralizing sera which have had considerable clinical employment in France. Recently Weyer, Park and Banzhaf (13) and Fairbrother (14) have reported more significant successes with the horse. The former found that the inactivating substances in the serum could be concentrated in the globulin fraction.

Through the kindness of Dr. Park, a quantity of antipoliomyelitic horse serum has been made available to us for study. We have carried through two series of experiments with this serum: inactivating effects in one *in vitro* and in the other *in vivo* have been investigated. It still remains to be determined whether the biological processes in both kinds of inactivation or neutralization are the same; and it is still an open question whether the inactivation which occurs with horse or sheep antipoliomyelitic serum is both qualitatively and quantitatively identical with that produced with convalescent human and monkey serum.

Considerable diversity of view exists with reference to the neutralizing power and process of these alien sera. Rosenow (15) and Nuzum (16) early claimed to have produced antiviral sera in the horse by immunization with streptococci. Amoss and Eberson (17), using serum supplied by the experimenters named, were unable to confirm this work. Stewart and Haselbauer (18) studied Pettit's antipoliomyelitic horse serum and found that it is occasionally virus neutralizing. They also found normal sheep serum to be sometimes inactivating, and reported that sheep yielding this serum when subjected to subsequent immunization not only failed to give a more potent serum, but occasionally lost the power of inactivation originally possessed.

Obviously, therefore, the nature of the inactivating process as exhibited by the antipoliomyelitic horse or sheep serum is worthy of more minute study. It is desirable also to employ a virus of definite and certain activity and preferably, we believe, a virus which possesses these powers when used as a filtrate rather than as suspension or

EXPERIMENTAL STUDY OF A HORSE ANTIPOLIO-MYELITIC SERUM

By C. P. RHOADS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Up to a recent date two sources of antipoliomyelitic serum have been known: one, human beings who have recovered from attacks of poliomyelitis irrespective of their severity; and the other, monkeys, experimentally inoculated with the virus of the disease. Monkeys, like human beings, were found to yield a virus neutralizing serum, not only when they developed symptoms, usually paralytic, of poliomyelitis, but also when they were actively immunized with subclinical doses of the virus itself. There is reason to believe that such subclinical immunization takes place in human beings, perhaps on a large scale, during epidemic prevalences of poliomyelitis.

The knowledge summarized in this brief statement is based on the investigation carried out in 1910 and since then (Flexner and Lewis (1), Roemer and Joseph (2), Landsteiner and Levaditi (3), Zappert, von Wiesner, and Leiner (4), Anderson and Frost (5), Amoss and Chesney (6), and Aycock and Kagan (7)). In 1910, Flexner and Lewis (9) determined definitely, by monkey experiment, that convalescent monkey and human sera possessed therapeutic properties. Netter (8) was the first to apply this knowledge to the treatment of human cases of poliomyelitis. The experiments of Flexner and Lewis (9), carried out with potent virus inoculated intracerebrally, indicated that with a minimal but effective dose of virus, and several intrathecal injections of convalescent serum begun 24 hours after the inoculation, prevention of paralysis or other symptoms of poliomyelitis could be secured. In the years intervening between 1910 and the present time, convalescent human serum has been widely employed in the treatment of early cases of poliomyelitis in man with beneficial results, so far as clinical and statistical indications can be interpreted.

Attempts were early made by several experimenters to produce a virus neutralizing serum in animals other than monkeys.

Flexner (10) in 1910 reported negative results with horse, goat, and several kinds of small laboratory animals. Sheep alone yielded inactivating substances.

Comment.—Taken by itself, this *in vitro* experiment has reproduced the usual experience with convalescent human and monkey sera.

Experiment 2.—This test was an orienting one, designed to indicate the limits of effectiveness possessed by the antipoliomyelitic horse plasma. Consequently

TABLE I

Experiment 1. Unpreserved Antipoliomyelitic Horse Serum

No.	Date	Material tested	Amount	Virus amount	Route of inoculation	Results
			cc.	cc.		
1	5/20	Unfiltered, unpreserved antipoliomyelitic serum	0.9	0.3	Icer.	No symptoms
2	5/20	"	0.9	0.3	"	"
3	5/20	Normal, unpreserved horse serum	0.9	0.3	"	Typical poliomyelitis on 9th day

TABLE II

Experiment 2. Antipoliomyelitic Horse Plasma

No.	Date	Material tested	Amount	Virus amount filtrate	Result
			cc.	cc.	
1	8/23	Normal horse serum. Control	0.1	0.01	Typical poliomyelitis, 20 days
2	8/23	Normal horse plasma. Control	0.1	0.01	No symptoms
3	8/23	Antipoliomyelitic horse plasma	0.1	0.06	Mild symptoms, 12 days
4	8/23	"	0.1	0.12	No symptoms
5	8/23	"	0.1	0.18	Typical poliomyelitis, 4 days

the volume of infecting filtrate was varied widely, and the relations between virus and plasma were so arranged as to comply as closely as possible with the technique used by Park and Weyer. The virus calculated on its usual activity represented

emulsion. The very active filtrates are more constant in their effects, and the effective dose can be more closely regulated. It is self-evident that the contact between serum and filtrate virus will be more intimate than between serum and emulsion virus, in which relatively large particles of nervous tissues must be penetrated.

Small differences in incubation period in the inoculated monkeys cannot be regarded as significant. Any large series of virus inoculated monkeys will show disparities of this kind, even when intracerebral injection is employed. This is to be expected, since the native resistance of the animals to a fixed inoculum varies within certain limits. Flexner and Lewis (19) (1910) pointed out that with a given virus, four kinds of inoculation effects could be distinguished: no discernible symptoms of disease; abortive poliomyelitis; paralytic poliomyelitis on about the 10th day; and delayed paralytic poliomyelitis in which the first symptoms appeared on about the 15th day. The first two conditions were distinguishable on reinoculation: the abortive animals resisted; the symptomless animals reacted to the second inoculation. In rare instances, paralytic effects appeared as late as the 28th day. Hence 1 month was always permitted to elapse before the experiment was considered concluded.

EXPERIMENTAL

In the experiments to be recorded the highly potent filtrate virus "M.V." has been employed. Injections were made into *Macacus rhesus* alone. The anti-poliomyelitic horse sera kindly supplied by Drs. Park and Weyer consisted of unpreserved horse serum, citrated horse plasma, and concentrated globulin fraction. The controls employed consisted of normal horse serum, citrated horse plasma, and the globulin fraction of diphtheria antitoxin. All inoculations which entailed surgical procedures were carried out under ether anesthesia.

Experiment 1.—This test is an exact duplicate of tests frequently made with convalescent human and monkey sera. The virus filtrate, consisting of 0.3 cc., is mixed with serum 0.9 cc., incubated at 37°C. for 2 hours, allowed to stand over night in the icebox, and the whole is injected intracerebrally. Under these circumstances, the immune sera neutralize the virus, while the normal horse and strictly normal human sera do not.

Three monkeys were employed: one received normal, unpreserved horse serum (control), and the other two received the unpreserved antipoliomyelitic horse serum. The inoculations were intracerebral. Table I gives the results. The control animal became paralyzed on the 9th day; the two animals receiving the antipoliomyelitic horse serum remained free of all symptoms.

Experiment 3.—This test was made with the globulin concentrate, and controlled with normal horse plasma and antipoliomyelitic horse plasma, 2 M.L.D. of virus being employed in each test. The results are given in Table IV.

Comment.—Under the conditions of the experiment, all the antipoliomyelitic horse preparations inactivated the virus, while both the normal plasma and serum control animals became paralyzed on the 9th day.

Since the experiments of certain previous investigators and the recent tests of Weyer, Park and Banzhaf all indicate that by the

TABLE IV
Experiment 3. Horse 5 Globulin

No.	Date	Material tested	Amount	Virus amount	Result
1	11/18	Filtered antipoliomyelitic horse globulin	cc. 0.1	cc. 0.2	No symptoms
2	11/18	Unfiltered antipoliomyelitic horse globulin	0.1	0.2	" "
3	11/18	Antipoliomyelitic horse plasma	0.1	0.2	" "
4	11/18	Normal horse plasma	0.1	0.2	Typical poliomyelitis, 9 days
5	11/18	—	—	0.2	"

injection of poliomyelitic nervous tissues, an occasional horse may be made to develop inactivating substances for the virus effective *in vitro*, the tests which we have made may be regarded as confirmatory. We proceeded, therefore, to our next problems which related first, to the mechanism of the inactivation process, and second, to the *in vivo* effects of the antiserum.

The next experiment deals with the question of the action of adsorbing bodies on the inactivating substances. Andervont and Lewis (20) have shown that colloidal particles, such as aluminium hydroxide, kaolin, and India ink, adsorb vaccine virus and thus render it in-

12 to 36 infecting doses. The controls consisted of normal horse plasma and serum.

Comment.—As Table II shows, normal horse plasma is effective in preventing infection under conditions in which normal horse serum has no final effect. On the other hand, in one of three monkeys the antipoliomyelitic horse plasma was protective against at least 12

TABLE III
Experiment 2. Effect of 1.5 Per Cent Citrate on Poliomyelitis Virus

No.	Date	Material tested	Virus amount	Test material amount	Result
1	8/23	Citrated horse plasma	cc.	cc.	No symptoms
2	8/23	Uncitrated horse serum	0.01	0.1	
3	10/10	Citrated horse plasma	0.01	0.1	Typical poliomyelitis, 19 days
4	10/10	Uncitrated horse serum	0.01	0.1	Mild poliomyelitis, 9 days
5	10/17	Citrated horse serum	0.01	0.1	Typical poliomyelitis, 5 days
6	10/17	Uncitrated horse serum	0.01	0.1	No symptoms
7	11/1	Citrated horse plasma	0.01	0.1	Typical poliomyelitis, 6 days
8	11/1	Control virus alone	0.01	—	No symptoms
					Typical poliomyelitis, 7 days

M.L.D. of virus, and ineffective against 6 and 18 M.L.D. The incubation period of the monkey receiving normal horse serum was somewhat prolonged, the reason for which is wholly conjectural. Since the citrate itself may have exerted an inhibitory effect, a set of tests was carried out to determine this point. The result is given in Table III and can be summarized by the simple statement that sodium citrate in concentration of 1.5 per cent does exert an inhibitory chemical effect on the filtered virus.

employment of Pettit's serum for therapeutic purposes in man is postulated on these same properties. Especial interest, therefore, attaches to this class of experiments.

The experience of this laboratory with experiments of this kind is considerable. At the beginning the virus inoculations were made intracerebrally, obviously a severe test for the protective or therapeutic action of an antiserum. Later, intracisternal injection of the virus was employed in order to avoid the traumatic effects of intracerebral injection. More recently, intranasal instillation of virus has superseded the intracisternal method, as involving no trauma whatever and as employing the usual portal of entry of the virus in man.

The observations of Flexner and Stewart (21) on the duration of the protective effects of convalescent sera when injected intraspinally, which observations have been confirmed and extended by ourselves (22), indicate a noteworthy manner of approach towards solving the problem of the nature of the inactivating substances in the antipoliomyelitic horse sera. For instance, it had been shown by Flexner and Stewart and by ourselves that when 2 cc. of convalescent human or monkey serum is injected intrathecally, it is effective for 4 days against a subsequent intracerebral injection of virus; and we found that it is sometimes effective for 6 days against nasal instillation of the virus. In the next experiment, antipoliomyelitic horse globulin was substituted for the convalescent sera.

Experiment 5.—Two series of animals were employed in this experiment. 2 cc. of the antipoliomyelitic horse globulin was injected intraspinally by lumbar puncture, followed 4 days later by intracerebral injection of 1 M.L.D. of virus.

Comment.—As Table VI indicates, under conditions in which convalescent human and monkey sera prevent the development of experimental poliomyelitis, the antipoliomyelitic horse globulin solution was without demonstrable effect. The two sets of tests made are consistent. The monkeys given the intraspinal injections of serum exhibited symptoms of experimental poliomyelitis after the injection of virus in average incubation time. Attention should be called to Monkey 2 (Table VI), in which the symptoms are described as being mild. For the purposes of comparison, brief clinical descriptions are appended of the three monkeys employed in this series.

effective on inoculation. A simple test brought out the fact that the antipoliomyelitic horse serum contained precipitin for normal monkey brain proteins. Hence equal amounts of the antipoliomyelitic horse globulin and 5 per cent saline suspension of normal monkey brain were mixed and shaken for 2 hours, after which they were kept in a water bath at 37°C. for 2 hours and placed in the icebox over night. The clear fluid, separated by centrifugalization, was pipetted off. This fluid failed to give the precipitin reaction.

Experiment 4.—2 M.L.D. of the filtrate virus were mixed with 0.1 cc. each of unfiltered antipoliomyelitic horse globulin absorbed and unabsorbed with normal monkey brain, controlled by a simple virus injection.

TABLE V
Experiment 4. Normal Brain Absorption

No.	Date	Material tested	Amount	Virus amount	Result
1	11/29	Antipoliomyelitic horse unfiltered globulin absorbed by normal brain	cc. 0.1	cc. 0.2	No symptoms
2	11/29	Antipoliomyelitic horse unfiltered globulin unabsorbed	0.1	0.2	" "
3	11/29	Plain control	—	0.2	Typical poliomyelitis, 7 days

Comment.—As Table V shows, the monkeys injected intracerebrally with the globulin-virus mixtures remained symptomless, while the control animal became paralyzed on the 7th day. In other words, the inactivating substances contained in the antipoliomyelitic horse globulin were not removed by the absorption with normal monkey brain, as carried out in the experiment.

We turn now to what may be termed the crucial experiments, in which the antipoliomyelitic horse serum is compared in its effects with convalescent human and monkey poliomyelitic sera. Weyer, Park and Banzhaf, as well as Neustädter and Banzhaf, have described *in vivo* tests in monkeys in which the antipoliomyelitic horse serum preparations displayed protective and therapeutic properties. The

scribed. No globulin was injected, but the animal received the same intracerebral inoculation of active virus as did Monkeys 1 and 2. On the 10th day after inoculation, a marked tremor was present, with wild excitement, very marked ataxia, and weakness of the left arm. The following day, prostration had set in and the monkey was etherized. The lesions found were characteristic of poliomyelitis.

Experiment 6.—The next series of tests may be called therapeutic, in distinction from the immediately preceding tests, which may be called prophylactic. In the therapeutic series, intranasal instillation of the virus was employed for inoculation, and intraspinal injection of the globulin solutions for treatment. The series was controlled not only in the usual way, but with antidiphtheria globulin solution. This experiment can be given adequately in the form of a table (Table VII) which presents all the salient facts.

Comment.—This test is informing. The antipoliomyelitic horse globulin given intraspinally to three monkeys, 3 days after nasal instillation of virus, protected one animal completely, one animal doubtfully (excitement and ruffled fur for 2 days), and one animal not at all. Antidiphtheria globulin administered in the same manner seems to have protected both monkeys to which it was given, as neither developed symptoms of any kind. The precise interpretation of the experiment may be in doubt, and the tests call perhaps for repetition on a larger scale. The markedly inconstant protective effects of the antipoliomyelitic horse globulin are, however, in sharp contrast with the regular effects of convalescent sera (22) under similar conditions of experiment. They do not agree wholly with the reported statement of Weyer, Park, and Banzhaf (13) that the globulin solution injected intraspinally confers immunity of considerable duration against the virus instilled into the nares. We would suggest that the wide differences between Weyer and Park's experimental results and those we have secured are due, not to technical irregularities, but to the potency and certainty of pathogenic action of the strains of virus which were employed by us.

Experiment 7.—A final set of tests was carried out to determine the effect of Berkefeld filtration on the globulin solutions. The technique employed was the usual one of *in vitro* neutralization. The antipoliomyelitic horse globulin was found inactivating before filtration. Antidiphtheria horse globulin was employed for the control test. The results summarized in Table VIII are consistent—Berkefeld filtration deprived the globulin preparations of their inactivating properties.

Macacus rhesus 1 received 2 cc. of unfiltered globulin fraction of antipoliomyelitic horse serum, injected into the lumbar subarachnoid space by means of spinal puncture. 48 hours later the animal was inoculated intracerebrally with 0.1 cc. of fresh virus filtrate. After an incubation period of 9 days, the animal presented partial paralysis of one arm and complete paralysis of both legs. On the following day, complete paralysis of the extremities had set in, the respirations were shallow and rapid; the animal was etherized. Autopsy disclosed typical lesions of poliomyelitis.

TABLE VI
Experiment 5. *Prophylactic Effect of Antipoliomyelitic Horse Globulin*

No.	Date	Intraspinal globulin*		Interval before inoculation	Inoculations			Result
		Type	Amount		Virus filtrate	Route	Amount	
1	2/18	Antipoliomyelitic horse unfiltered globulin	2	4 days	M.V.	Icer.	0.1	Typical poliomyelitis, 9 days
2	2/18	"	2	4 "	"	"	0.1	
3	2/18	—	Control	—	"	"	0.1	Mild poliomyelitis, 7 days
4	3/24	Antipoliomyelitic horse unfiltered globulin	2	4 days	"	"	0.1	Typical poliomyelitis, 10 days
5	3/24	"	2	4 "	"	"	0.1	Typical poliomyelitis, 4 days
6	3/24	"	2	4 "	"	"	0.1	Typical poliomyelitis, 3 days
			2	4 "	"	"	0.1	Typical poliomyelitis, 4 days

* Injected by lumbar puncture.

Macacus rhesus 2. The animal received exactly the same spinal injection of antipoliomyelitic globulin and intracerebral inoculation of fresh filtrate virus as did Monkey 1. 7 days after inoculation, the animal displayed mild symptoms of illness consisting of excitement, fine tremor of the head, and ruffled fur—characteristics of early experimental poliomyelitis. The condition failed to progress further and the animal proved resistant to subsequent inoculation.

Macacus rhesus 3. This test served as a control for the two previously de-

TABLE VIII
Experiment 7. Effect of Filtration

No.	Date	Material tested for neutralizing power	Amount	Virus amount filtrate	Globulin virus ratio	Result
1	4/22	Antipoliomyelitic horse globulin-filtered	cc. 0.06	cc. 0.4	1:6	Typical poliomyelitis, 5 days
2	4/22	"	0.06	0.1	1:2	"
3	4/22	"	0.24	0.4	1:6	Typical poliomyelitis, 7 days
4	4/22	Control diphtheria horse globulin	0.06	0.1	1:2	"

SUMMARY

Through the kindness of Dr. W. H. Park we have been enabled to study a horse antipoliomyelitic serum. This preparation has been supplied us in three forms: citrated blood plasma, serum, and globulin concentrate.

We have tested these preparations *in vitro* and *in vivo* for inactivating or neutralizing or, to use perhaps a better term, antiviral effects against a constant, potent, filtrate virus of poliomyelitis.

The preparations exhibited these effects when combined *in vitro*. Their action in this respect appears to be greater and more constant than that found by Stewart and Haselbauer for the Pettit anti-poliomyelitic horse serum.

On the other hand, *in vivo* tests carried out by us were less successful. In comparison with the constancy of action, under given conditions, of convalescent monkey and human sera, the antipoliomyelitic horse serum displayed striking irregularity, and certain preparations were devoid of protective power.

The precise nature of the inactivating substances in the horse antiserum and their relation to the corresponding substances in convalescent sera have still to be determined. As far as one absorption test carried out by us indicates, precipitin does not play a major rôle in the inactivating process.

TABLE VII
Experiment 6. Therapeutic Action of Antipoliomyelitic Horse and Antidiphtheria Globulin

No.	Date	Inoculation				Globulin			Result
		Interval before serum	Route	Virus	Total amount	Method of inoculation	Type	Amount	Route
1	4/29	3 days	Nasal	M.V. 10% glycerolated	6 cc.	1 cc. each nostril daily for 3 days	Antipoliomyelitic horse filtered globulin	2	Spinal*
2	4/29	3 "	"	"	6	"	"	2	"
3	4/29	3 "	"	"	6	"	"	2	"
4	4/29	3 "	"	"	6	"	Diphtheria globulin	2	"
5	4/29	3 "	"	"	6	"	"	2	"
6	4/29	—	"	"	6	"	Control	—	"
									5/8. Typical poliomyelitis, 9 days
									No symptoms
									5/9. No symptoms
									"
									5/8. Typical poliomyelitis, 9 days

* Injected by lumbar puncture.

When an active globulin concentrate was filtered through Berkefeld candles, it lost its *in vitro* inactivating power. This is not true of convalescent sera in the native state. No tests have, however, been made with globulin concentrates from such sera.

The experiments described in this paper raise the question whether, therapeutically considered, the antipoliomyelitic horse serum should be regarded as an exact equivalent of, and hence employed as a perfect substitute for, convalescent serum. This question can only be answered by further experiment and observation.

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and the neutralizing value of serum obtained before and after the treatments was determined. The details of the experiment are as follows:

Reinforcement.—Six *Macacus rhesus* monkeys, which had survived typical attacks of experimental poliomyelitis and still showed residual paralyses 15 to 19 months after the original inoculation, were selected. Each was bled 20 cc., and the serum from the individual bleedings was separated in the usual manner. The several sera were pooled, and the resulting mixture was stored, unpreserved by chemicals, at 4°C. The “reinforcing” injections were begun immediately after the first bleeding and conducted in the following manner. A 5 per cent suspension of glycerolated nervous tissue of the “pooled mixed” virus strain was

TABLE I
Technique of Reinforcement

Monkey No.	Date of poliomyelitis	Treatment begun	Treatment ended	Virus	Concentration	Route of inoculation	Amount each treatment	Total number of treatments	Total amount of virus	Date of bleeding
1	8/23/29	11/2/30	12/3/30	Glycerolated M.V.	5% suspension	Intradermal	15 cc.	10	150 cc.	12/28/29
2	5/28/29	“	“	“	“	“	“	“	“	“
3	5/10/29	“	“	“	“	“	“	“	“	“
4	6/15/29	“	“	“	“	“	“	“	“	“
5	4/17/29	“	“	“	“	“	“	“	“	“
6	5/28/29	“	“	“	“	“	“	“	“	“

employed in physiological saline solution. The intradermal route of inoculation was selected, since Stewart and Rhoads (2) had shown that route to be more effective than subcutaneous inoculation for giving rise to an active immunity in monkeys. 15 cc. of the material was introduced in each set of injections by forming multiple superficial blebs. The treatments were repeated 10 times at 3 day intervals. Thus a total of 150 cc. of virus was given in 30 days. After a rest period of 1 month, the animals were bled, and the serum was separated, pooled, and stored as before. The monkeys were carefully observed to detect any evidence of recurring symptoms during the injections, and none whatever was seen. The technique of reinforcement is summarized in Table I.

Neutralization before Reinforcement.—In determining the effectiveness of the serum, the usual *in vitro* technique was employed. The fresh Berkefeld filtrate virus was mixed with the serum to be tested, kept an hour at 20°C., and inoculated intracerebrally into normal monkeys of approximately the same size.

REINFORCEMENT OF CONVALESCENT ANTIPOLIO- MYELITIC SERUM IN THE MONKEY

By C. P. RHOADS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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Flexner and Lewis (1), in connection with their studies of the protective and therapeutic powers of convalescent serum in experimental poliomyelitis, employed the method of reinoculation of virus in recovered monkeys to increase the strength or potency of this serum. The procedure was largely empirical, as no actual quantitative estimations of neutralizing action before and after the reinoculations were made. The impression gained, however, was that reinoculation did increase the potency of the serum.

Monkey immune serum as now employed for experimental purposes may be considered to be of two sorts: convalescent serum in the true sense, and serum derived from monkeys actively immunized with virus introduced by various routes without symptoms of infection appearing at any time. In practical experiments, little or no distinction has been made between these two kinds of immune sera, and no exact quantitative neutralization comparisons have been carried out. Stewart and Rhoads (2) have, however, shown that monkey serum may be virus neutralizing *in vitro* when the actively immunized animals yielding it are incapable of withstanding an intracerebral injection of a highly potent virus. This experimental discrepancy between the *in vivo* and *in vitro* inactivating power of immune sera is instructive in that it not only indicates quantitative variations in immune power, but suggests that human beings also may yield neutralizing serum without themselves being completely or enduringly protected against the pathogenic action of a highly infectious virus strain.

In order to decide whether or not quantitative differences in various immune sera actually existed, a simple experiment was carried out. Monkeys which had recovered from typical poliomyelitis were repeatedly reinoculated with large amounts of active poliomyelitis virus,

the pooled reinforced serum contained greater quantities of neutralizing antibodies than did the original pooled convalescent serum from the same monkeys. The fact is even more striking in that, so far as the tests were carried, there is clear indication that the reinforced serum possessed neutralizing value equal to that of pooled convalescent human serum. A discrepancy will be noted between the series of animals inoculated March 5, 1930, and the experiment summarized in Table III; in the former instance 0.5 and 0.25 cc. of

TABLE III
Neutralizing Value of Monkey Convalescent Serum before Reinforcement

Date	No.	Serum		Virus		Route	Preliminary treatment	Result	Incubation period
		Type	Amount	Strain	Amount filtrate				
10/30	1	Monkey convalescent serum before reinforcement	cc. 0.5	M.V.	0.12	Icer.	1 hour 20°	Typical poliomyelitis	14 days
10/30	2	"	0.75	"	0.12	"	1 hour 20°	No symptoms	—
10/30	3	"	1.0	"	0.12	"	1 hour 20°	" "	—
10/30	4	Pooled human convalescent	0.1	"	0.12	"	1 hour 20°	" "	—
10/30	5	Control	0.1	"	0.12	"	1 hour 20°	Typical poliomyelitis	7 days

non-reinforced serum protected against 0.12 cc. of virus filtrate, although 0.1 cc. failed to do so. The probable reason for this difference is to be found in the virus filtrate. A degree of inconstancy is encountered even in dealing with the most highly potent virus strains, for which adequate explanation is not at hand. The filtrate prepared from an occasional monkey, sacrificed promptly after the appearance of paralytic symptoms, proves somewhat less active than the rule. Whether the fault is due to the quantity of virus units in the nervous

Tables II and III are consistent in showing that, given a constant potent virus filtrate used in amounts of 0.12 cc., the pooled convalescent monkey serum before reinforcement was ineffective in quantities less than 0.75 to 1 cc. A sample of pooled human convalescent serum in a volume of 0.1 cc. neutralized effectively in one test (Table III).

Although the meaning of the test is not at once clear, it is well worth recording that in two instances a single sample of a human serum, taken from a child 8 years old who had never shown clinical evidence

TABLE II
Neutralizing Power of Monkey Convalescent Serum before Reinforcement

Date	No.	Serum		Virus		Route	Preliminary treatment	Result
		Kind	Amount	Strain	Amount filtrate			
8/30	1	Monkey immune, non-reinforced	0.05	M.V.	0.12	Icer.	1 hour 20°	Typical poliomyelitis, 5 days
8/30	2	"	0.1	"	0.12	"	1 hour 20°	Typical poliomyelitis, 7 days
8/30	3	"	0.5	"	0.12	"	1 hour 20°	Typical poliomyelitis, 11 days
8/30	4	"	1.0	"	0.12	"	1 hour 20°	No symptoms
8/30	5	Control	—	"	0.12	"	1 hour 20°	Typical poliomyelitis, 5 days

of poliomyelitis, effected neutralization in the proportion of serum 0.1 cc. and virus filtrate 0.12 cc. This observation is in conformity with earlier experiments of Anderson and Frost (3), in which supposedly normal human serum was found to be inactivating, and with recent tests by Aycock and Kramer (4). The latter attribute the inactivating power of the serum to nonclinical mass immunization to the virus of poliomyelitis.

Neutralization after Reinforcement.—Table IV, which includes three separate tests made on different dates, presents clear evidence that

human serum, either as a prophylactic or therapeutic measure, it may be desirable to employ not merely convalescent, but reinforced convalescent monkey serum. If serum from actively immunized monkeys is also employed, preliminary tests of neutralizing power are desirable. Undoubtedly certain discrepancies and failures of experiments are traceable to the use of weak convalescent monkey serum instead of the stronger human convalescent serum. For experiments on monkeys the homologous reinforced monkey serum may be desirable.

SUMMARY

A comparison has been made of the neutralizing value of pooled convalescent monkey serum for the filtered virus of poliomyelitis, before and after a series of reinforcement injections of the same virus strain.

The strength of the pooled convalescent serum is increased by the reinforcing procedure.

The original monkey convalescent serum had a neutralization value much below that of a pooled human convalescent serum. By reinforcement the neutralization value of the monkey serum was brought approximately to that of the human serum.

One sample of serum from a supposedly normal child of 8 years exhibited a neutralizing value approximately equal to that of a pooled human convalescent serum and the reinforced pooled monkey serum.

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system of a particular animal, or is influenced adversely by the operation of extracting it, is not known. On the whole, however, this series of experiments was remarkably regular.

TABLE IV
Comparative Neutralizing Power of Monkey Convalescent Serum before and after Reinforcement

Date	No.	Serum		Virus		Route	Preliminary treatment	Result
		Type	Amount	Strain	Amount filtrate			
2/13	1	Reinforced monkey convalescent	0.5	M.V.	0.12	Icer.	1 hour 20°	No symptoms
2/13	2	"	0.1	"	0.12	"	1 hour 20°	" "
2/13	3	Control	—	"	0.12	"	1 hour 20°	Typical poliomyelitis 5 days
3/20	4	Before reinforcement	0.5	"	0.12	"	1 hour 20°	Typical poliomyelitis 11 days
3/20	5	"	0.1	"	0.12	"	1 hour 20°	Typical poliomyelitis 6 days
3/20	6	After reinforcement	0.5	"	0.12	"	1 hour 20°	No symptoms
3/20	7	"	0.1	"	0.12	"	1 hour 20°	" "
3/20	8	Control	—	"	0.12	"	—	Typical poliomyelitis 12 days
3/5	9	Before reinforcement	0.5	"	0.12	"	1 hour 20°	No symptoms
3/5	10	"	0.25	"	0.12	"	1 hour 20°	" "
3/5	11	"	0.1	"	0.12	"	1 hour 20°	Typical poliomyelitis 9 days
3/5	12	After reinforcement	0.5	"	0.12	"	1 hour 20°	No symptoms
3/5	13	"	0.25	"	0.12	"	1 hour 20°	" "
3/5	14	"	0.1	"	0.12	"	1 hour 20°	" "

There is a practical side to these observations. Hereafter in testing the prophylactic and therapeutic value of convalescent monkey serum for the purpose of securing indications of the value of convalescent

The pericardial sac is a fibrous bag, very impermeable and very inactive in providing for removal of foreign material. When fluids or particles are placed in the pericardial cavity, the absorbing surface also includes the heart and the short intrapericardial lengths of the great blood vessels. In the areolar tissue immediately below the epicardial serosa, both upon the heart and upon the great vessels, there is a rich plexus of lymphatic capillaries which communicates with the lymphatics in the heart muscle and eventually with the subendocardial lymphatic plexus. All of these vessels drain into the large trunks which pass to the same nodes at the base of the heart as are entered by the pericardial lymphatics. In addition to the absorptive routes offered by the scanty supply of lymphatic capillaries in the pericardium and the larger number in the epicardium, there are the blood capillaries in both areas. Blood vessels are not numerous in the pericardium but are thoroughly so in the epicardium.

As a result of many different groups of experiments one may expect that lymphatics will be the paths for removal of particulate material and of solutions such as the blood proteins. The blood capillaries, on the other hand, will provide for rapid absorption of water and salts of small molecular size. Entrance to lymphatics is both indirect and direct. If particles of carbon or other foreign material are deposited in the neighborhood of lymphatic capillaries, phagocytosis begins fairly promptly and phagocytic cells carrying particles migrate into the lymphatics. This indirect method of entrance is exceedingly slow. On the other hand, many observations have shown that motion is extremely important, not only for getting lymph to move along lymphatics but also for bringing about the direct entrance of material into the lymphatic capillaries. Thus, Macallum (6) observed the effectiveness of diaphragmatic movements in causing particles in the peritoneal cavity to enter the lymphatics of the central tendon. Recently Florey (7) has found that if India ink was injected into the thigh muscles of a rat, and these muscles then tetanized at second intervals for 20 minutes, the glands at the bifurcation of the aorta became filled with carbon and the lymph trunk running along the back of the femoral vessels was jet black. When the same experiment was done without activity the ink remained localized, and removal depended upon the indirect phagocytic method.

ABSORPTION FROM THE PERICARDIAL CAVITY

By CECIL K. DRINKER, M.D., AND MADELEINE E. FIELD

(From the Department of Physiology, Harvard School of Public Health, Boston)

PLATES 5 AND 6

(Received for publication, September 20, 1930)

While engaged in a general analysis of the functions of the lymphatic system and the mechanism of lymph movement, we were surprised to find little information upon absorption from the pericardial sac. Hamburger (1) made five experiments on the pericardium of the dog, using salt solutions and horse serum. He found slow removal of serum. His observations were not concerned with possible routes of absorption and do not apply to our interest. Coupled with lack of data on absorption is lack of an adequate description of pericardial lymphatics.

Schumkow (2) described somewhat sketchily two sets of lymphatics in the pericardium of the dog and calf. If he excised the pericardial sac and filled it first with salt solution and later with Berlin blue, he had no trouble in making the dye flow into these vessels. His colleague, Skworzow (3) very briefly confirmed his findings and declared that the dye got into the lymphatics through stomata such as von Recklinghausen had declared existed on the peritoneal surface of the central tendon of the diaphragm.

Our experiments have been made entirely upon the rabbit. In this animal the pericardium is exceedingly thin, very like the omentum except toward the base of the heart where it becomes heavily loaded with fat. We have never found lymphatics in the thin, transparent pericardium, though they are abundant in the fatty tissue at the base of the heart and where lines of fat extend down upon the pericardium. They drain into several small nodes embedded in the basal cardiac tissue. It is quite possible that the transparent parts of the pericardium do contain a few lymphatics just as is probably the case for the thinnest parts of the omentum (4, 5); but they are certainly not numerous, and this is equally true of the blood vessels.

Absorption of Salt Solution

One series of experiments was done to determine the rate and manner of absorption of a 1.6 per cent solution of methylene blue in Ringer's solution. The solution was injected at such a rate that there was no appreciable change in the venous pressure which was simultaneously recorded. 0.5 cc. of solution was injected every 5 or 10 minutes for a period of about an hour. Table 1 shows the amount and rate of absorption of Ringer's solution from the rabbit pericardium. The average rate was about 1.3 cc. per hour.

The appearance of the heart in all these experiments was striking. The auricles and great vessels were very deeply stained and the thin right ventricle presented a marked contrast to the left ventricle. Quite frequently the staining of the ventricles would be concentrated in the groove between the two ventricles or would be in patches on the anterior side. The pericardium itself stained lightly but uniformly. The right and left ventricles, when slit open, showed no blue staining on the inside, whereas the interior of the auricles down to the coronary sulcus was quite deeply and distinctly stained. This would seem to indicate that the thinner parts of the heart, the parts nearer the base of the heart and under low pressures, are most easily penetrated by pericardial fluids. Fig. 1, A and B, shows typical hearts. The protocol of a typical experiment from which Fig. 1-A was drawn follows:

March 31, 1930. Absorption of a 1.6 per cent solution of methylene blue in Ringer's solution. Normal rabbit. Weight 2.6 kg. 8:45 a.m., 24 cc. 10 per cent sodium barbital intraperitoneally for anesthesia. 10:30 a.m., operation begun. Chest opened without injury to either pleural cavity. 11:00 a.m., pericardium cannulated. 11:03-12:25 a.m., 4.5 cc. of solution injected at 10-minute intervals. Venous pressure at the beginning, 2.5 cm. of water; at the end, 2.0 cm. of water with no appreciable change at any time during the period of injection. 1:15 p.m., autopsy. 1.0 cc. of blue solution recovered from the pericardial cavity. Heart stained in characteristic manner (Fig. 1-A). Lymph nodes in fat around the base of the heart stained blue.

Samples of urine, taken from the rabbits at the conclusion of the experiment, were usually tinged with blue. Lymph nodes in the fat at the base of the heart were occasionally found to be stained blue. The staining of the heart, however, indicates that much of the absorption of a simple salt solution from the pericardial cavity of the

In the face of such observations as these, one would expect that particles or serum in the pericardial cavity subjected to the pounding and churning of the heart would enter lymphatics rapidly and be carried promptly to adjacent lymph nodes. Much to our surprise this did not prove to be the case. Lymphatic absorption from the pericardial sac is an extremely sluggish process.

EXPERIMENTAL

Technique.—In spite of the extreme delicacy of the pericardium of the rabbit, these animals were employed since it is possible to expose the pericardium without entering either pleural cavity because of the widely separated and complete mediastinal partitions. Artificial respiration is unnecessary and animals can live for 2 hours with pericardium exposed. Sodium barbital was the anesthetic in terminal experiments and ether in recovery experiments. The pericardium was exposed through a small opening made on the left side and as near to the sternum as possible. After resection of 1 cm. of the third rib, the intercostal muscles and membranes between the lower border of the second and the upper border of the fourth ribs were cleaned away. The mammary vessels, being at some distance from the sternum, could usually be sufficiently retracted to make ligaturing unnecessary. Fat above the heart and on the pericardium was dissected off and a good view of the heart in the pericardium was obtained.

Material was injected into the pericardium by means of a syringe with a No. 26 hypodermic needle. In those experiments in which injections were to be made from time to time, a blunt syringe needle was inserted into the pericardial cavity, care being taken not to touch the heart. This was held in place with a hemostat and the syringe was in turn held by an adjustable clamp. Measured injections were made by pushing in the plunger of the syringe.

The jugular vein was cannulated and the venous pressure was recorded by means of a water manometer. Measurements were made simultaneously with the injections.

In those experiments in which the material introduced into the pericardium was to remain for some time, all of the usual precautions for asepsis were taken. A piece of the pericardium was picked up with a hemostat, the syringe needle inserted, and the injection made. After withdrawing the syringe, the small hole in the pericardium was ligatured securely beneath the tip of the hemostat. The wound was then sewed up, and the animals recovered rapidly from the operation. All of them remained in excellent condition.

At the conclusion of the experiment, the animal was bled to death through the carotid artery and a very thorough autopsy was performed on the thorax. The pericardium was opened and whatever liquid remained in the cavity was withdrawn by means of a pipette.

the simple salt solution. The epicardium over the left ventricle was only very lightly stained, if at all; and the thinner parts of the heart, the auricles, the tissue around the great vessels, and the right ventricle were stained, but far below the values obtained with the same dyes in physiological salt solution. In no case was there any staining of the pericardium.

Graphite.—A third series of experiments was done to determine the manner and path of absorption of particulate matter from the pericardial cavity. For this purpose a sterile solution of graphite suspended in salt solution (9) was employed. From 1 to 2 cc. of this suspension diluted with salt solution was introduced into the pericardial cavity and allowed to remain there for lengths of time varying

TABLE 2

Absorption of Serum from the Pericardium of the Rabbit

Experiment	Amount injected	Amount recovered	Amount absorbed	Time
	cc.	cc.	cc.	hours
16	1.1	0.0	1.1	5.0
17	1.1	0.0	1.1	5.0
18	3.0	3.0	0.0	3.75
19	1.5	1.5	0.0	2.75
21	2.0	2.0	0.0	4.0
22	1.0	1.0	0.0	2.0

from 24 hours to 3 weeks. At the end of the chosen period, the animals were killed.

Almost invariably there was found a large amount of thick graphite-containing exudate of a fibrinous nature. This was tightly adherent to the heart in the longer-period animals—2 to 3 weeks. In short-period animals it appeared as a slimy, membranous exudate just inside the pericardium.

The pericardial exudate, as a result of the sterile irritant, graphite, is very characteristic. Large mononuclear phagocytes, such as are shown in Fig. 2-B, are very plentiful. These cells were invariably strongly phagocytic for graphite and, apparently with slight help from polymorphonuclear leucocytes, represent the normal means of getting the foreign material out of the pericardium. Sections of the heart invariably showed these large cells filled with graphite in the

rabbit is cardiac and vascular. The rate is slower than one would expect considering the large number of subepicardial capillaries.

One experiment, done on the cat, indicates that most of the absorption of simple solutions from the pericardial cavity must be cardiac.

The chest of the anesthetized cat was opened under artificial respiration, the pericardium slit open and the edges sewed to the sides of the chest, thus making it absolutely tight (8). A glass oncometer was slipped over the heart, the thin rubber membrane resting at the auriculoventricular sulcus, only the ventricles being contained within the oncometer. The hole through the membrane was sufficiently loose so as not to constrict the vessels. 6 cc. of Ringer's solution and methylene blue were introduced into the oncometer. At the end of 2 hours, 2 cc. of solution

TABLE 1

Absorption of Physiological Salt Solution from the Pericardium of the Rabbit

Experiment	Amount injected	Amount recovered	Amount absorbed	Time	Rate of absorption per hour
	cc.	cc.	cc.	hours	cc.
4	3.0	0.0		2.0	1.5
5	7.0	5.0	3.0	2.0	1.0
6	5.0	1.25	2.0	2.0	1.88
7	5.0	1.75	3.75	2.0	2.5
8	5.0	3.0	3.25	1.33	1.33
9	3.5	3.25	2.0	1.5	0.25
11	4.5	2.25	0.25	1.0	1.1
12	4.5	1.0	2.25	2.0	1.75
13	5.0	1.0	3.5	2.0	0.5
			4.0	2.0	

were recovered, indicating the absorption of 2 cc. an hour by the heart itself. The staining of the heart was very distinct, there being a very definite line of demarcation where the solution touched the heart. The two ventricles were well stained, but the thin right ventricle was much more deeply stained than the left. Urine, removed at the end of the experiment, was slightly bluish green in color.

Absorption of Serum

Table 2 shows the second series of experiments done to determine the absorption, if any, of normal serum introduced into the pericardial cavity. Both rabbit and horse serum, colored with methylene blue or trypan blue, were used. The absorption of the serum was negligible; in four out of six experiments there was none whatever. The staining of the heart in these cases was quite different from that observed with

2. Rabbit serum and horse serum are absorbed extremely slowly—an indication of the low-grade lymphatic drainage of the pericardial sac.

3. Graphite particles of bacterial dimensions are also removed very slowly. Such particles enter lymphatics only after phagocytosis. The lymphatics in the basal part of the pericardium are the principal source of drainage. Subepicardial lymphatics are entered with difficulty from the pericardial cavity.

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EXPLANATION OF PLATES

PLATE 5

FIG. 1, A and B. Distribution of dye on the outside and inside of rabbit hearts after injections of dye into the pericardial sac. Identical results were obtained with methylene blue and trypan blue.

PLATE 6

FIG. 2. A, mononuclear phagocytes from the normal pericardial fluid of a rabbit which had received graphite 24 hours before; supravital preparation; large round inclusions, stain; small black particles, graphite. B, typical field of exudate 5 days after graphite injection; Wright's stain; the large mononuclear phagocytes can be shown to take vital stain. Magnification, $\times 650$.

subserous areolar tissues—in certain instances definitely within lymphatics. Very little graphite was free, and that seen was most probably due to breakdown of phagocytes with release of their load. Spreads of the pericardium showed, in transparent areas, many of the same large mononuclear phagocytes, often completely covered by graphite so that they appeared as small black balls. They were tightly adherent to the surface but never appeared to be in vessels. Toward the base of the heart in the fatty tissue, graphite could be seen occasionally within lymphatics and was almost invariably intracellular. Fig. 2 illustrates typical phagocytes.

The large cardiac lymphatic trunks never contained enough graphite to be visible with a binocular dissecting microscope. That this should be true after 3 weeks time is strong evidence for the difficulty with which the cardiac lymphatics are entered from the pericardial cavity.

DISCUSSION

The pericardium in the rabbit proves to be a singularly inert protective membrane. Simple solutions placed within the sac are held without leakage and are absorbed practically entirely by the subepicardial blood capillaries. Such solutions do not leak through the extraordinarily thin pericardial membrane into the pleural cavities even if subjected to slight pressure. When substances such as serum or graphite are injected removal is extraordinarily slow. No evidence was obtained showing the abrupt, direct type of lymphatic entrance which is seen in the central tendon of the diaphragm after intraperitoneal injections. Such lymph drainage as occurs is through lymphatics in the pericardium around the base of the heart and to a slight extent along lines of fat deposition in the pericardium. The subepicardial lymphatics are entered with great difficulty from the pericardial sac, a condition favorable to exclusion of the heart from participation in pericardial infections.

SUMMARY

1. Physiological salt solution is absorbed from the pericardial cavity of medium-sized rabbits at a rate of approximately 1.3 cc. per hour. This absorption is via the subepicardial blood capillaries.

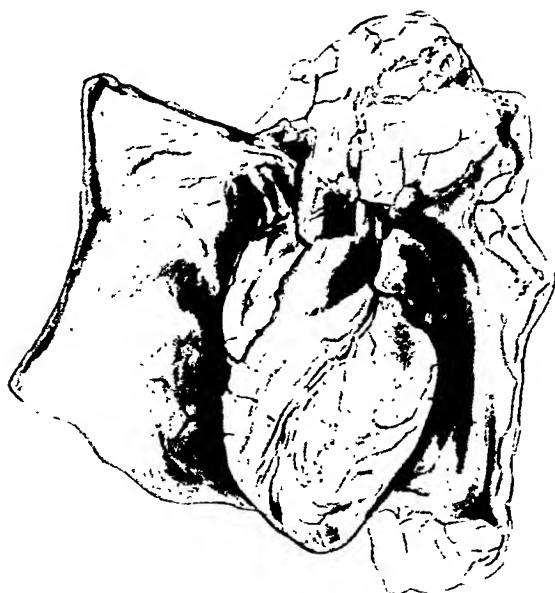


FIG. 1-A

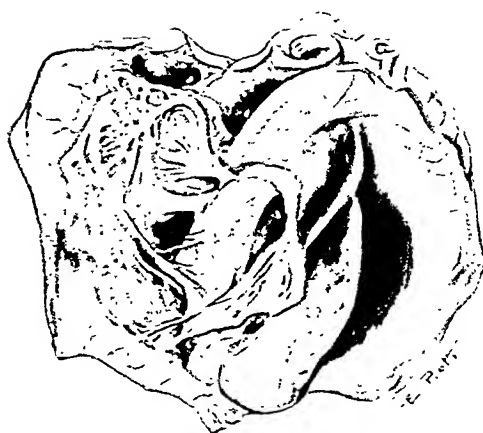


FIG. 1-B

(Drinker and Field: Absorption from pericardial cavity)





FIG. 2-A

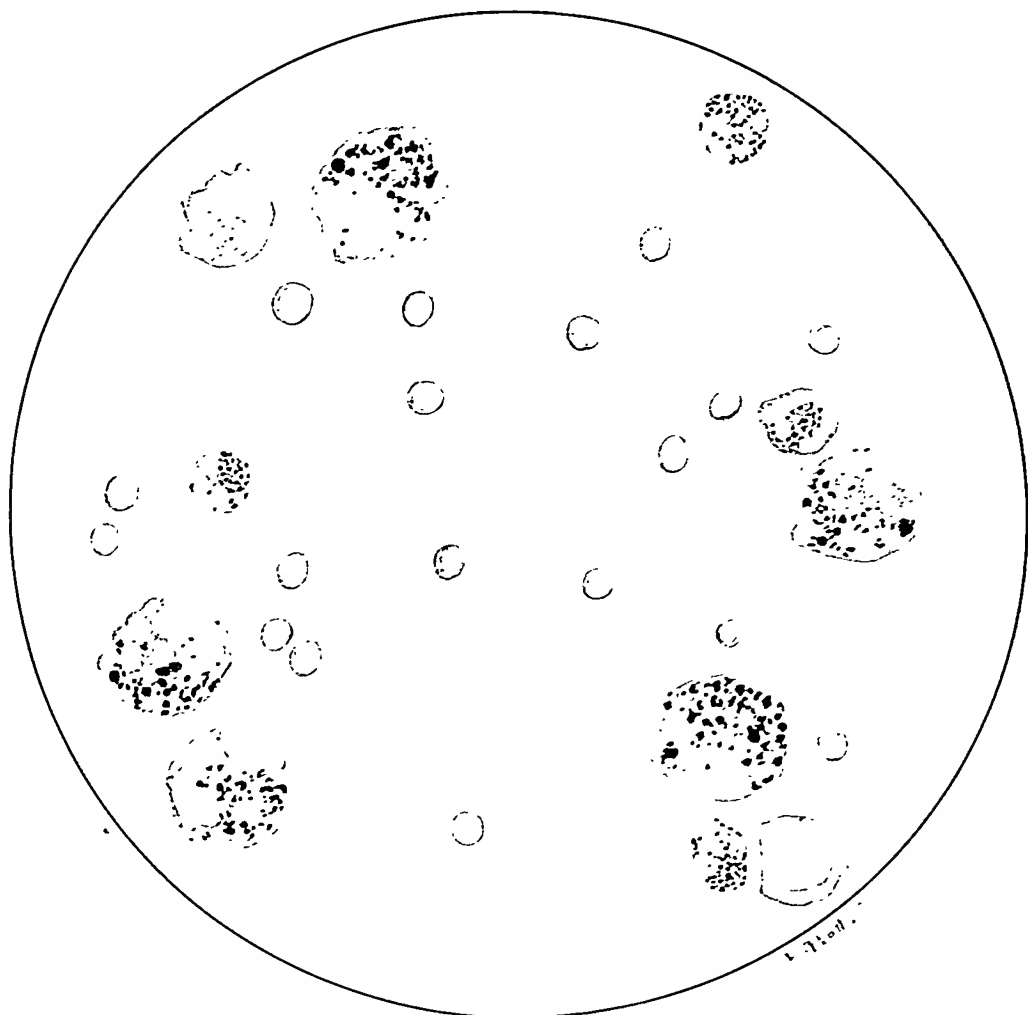


FIG. 2-B

(Drinker and Field: Absorption from pericardial cavity)

period of critical fall in the temperature. Our opportunity to investigate the early period of the disease has been limited. The serum from eight patients tested during the 1st or 2nd day showed no protective power. Protection was demonstrated as early as the 3rd day in three (one Type I, one Type II and one Type III) of eleven cases, and on the 4th day in five (four Type I and one Type III) of fourteen cases.

As shown in the accompanying chart (Chart 1) in one patient (Case 1, Type I) with protection on the 3rd day, the temperature fell to

Day of disease	1	2	3	4	5	6	7	8	9	10	11	12	13	14	21	27	59	67
Type I	1		105	+	+													
	2			72	180	183	+	+	+			+						+
	3			124	155	35	+	+	17	17	15	15	12			0		
	4			48	51	48	48	48										
	5			+	+		+				+				+			
Type II	1		120	+	+		+							0				
Type III	1	0	0	+	+		+	+	+	+	+	+	+	+			0	
	2			+		+	+		+									

CHART 1. Early appearance of antibody and its relation to time of recovery.

O = no mouse survivals. + = one or more surviving mice. \ = fall in temperature. ^ ^ = irregular temperature fall. The figures in the upper space show the number of thousands of Felton units of the corresponding type which were administered after the serum sample was taken.

normal on the 4th day and in another (Case 5, Type I) with protection on the 4th day the patient became afebrile the same day. The early appearance of protection is not, however, always followed by a speedy termination of the disease, as the two other patients (Case 1, Type II, and Case 1, Type III) with protection on the 3rd day remained febrile until the 6th while three (Case 3, Type I, Case 2, Type I, and Case 4 Type I) with protection on the 4th day had fever until the 7th, 9th and 10th day respectively, in spite of considerable amounts of Felton's antibody after the collection of the first blood sample.

CERTAIN ASPECTS OF MOUSE PROTECTION TESTS FOR ANTIBODY IN PNEUMOCOCCUS PNEUMONIA*

By FREDERICK T. LORD, M.D., AND ELBERT L. PERSONS, M.D.

(From the Medical Laboratory and Services of the Massachusetts General Hospital,
Boston)

(Received for publication, October 27, 1930)

In continuation of the study of antibody in lobar pneumonia 342
mouse protection tests have been done on 63 patients† as follows:

CORRECTION

In Vol. 53, No. 1, January 1, 1931, page 93, eighth line under the heading "Experimental," for "may be defined as the smallest amount of antiserum," read
"may be defined as ten times the smallest amount of antiserum."

Time of Appearance of Antibody and Its Relation to the Time of Recovery

In general in our series, as noted by Dochez (3) in his investigation, the time of appearance of antibody coincides rather sharply with the

* This investigation was made with assistance from the Proctor Fund of Harvard University, the Committee on Therapeutic Research, Council on Pharmacy and Chemistry of the American Medical Association and the American Association for the Advancement of Science.

† The material from 31 of these cases was kindly furnished by Drs. W. D. Sutliff and Maxwell Finland of the Boston City Hospital and from 9 cases by Drs. George Walker and Samuel Shelburne from the service of Dr. Henry A. Christian at the Peter Bent Brigham Hospital. We are indebted to Dr. Lloyd D. Felton for virulent cultures of the pneumococcus.

Irregular Protection Tests in Mice

Irregular protection tests are not uncommon in the investigations of others. Of a total of 379* mouse protection tests, 75 show scattered or irregular survivals of mice, and in 33 tests these irregularities are marked enough to make it impossible to estimate the approximate number of lethal doses of pneumococci against which the individual serum sample protected. We are unable to trace these sporadic survivals to any error in technic, and it seems probable that the survival of even one mouse, in the absence of technical error and in view of the death of the control mice, indicates the presence of some protective substance in the serum injected with the organisms.

Amount of Antibody in Untreated Cases

As noted by Dochez (3) the amount of protection demonstrable at the time of crisis, though variable, is small compared with the potency of sera obtained by active immunization of larger animals. Of 17 untreated cases in our series, tested within 1 day on either side of crisis, 12 (8 Type I, 1 Type II, 3 Type III) had protective power against 100 lethal doses or less of pneumococci of corresponding type and none of the remaining five (3 Type I, 2 Type II) had protective power against more than 10,000 lethal doses.†

Amount of Protection in Treated Cases

In attempting to estimate the effect of late intravenous specific treatment on the protective power of the patient's serum, the problem is complicated by the usual production during the course of the disease of small and varying amounts of protective substances by the infected individual himself.

Twenty-nine surviving cases, treated with Felton's antibody, had protection tests on 1 of the 3 days centering at the critical fall in temperature. Of these, eleven (all Type I) showed protective power

* This figure includes a miscellaneous group of 37 protection tests which has not been included in the series, consisting of cases on which only one test was done, cases in which a reliable history was lacking, Type IV cases which were tested against types for which antibody had been administered and the like.

† The equivalent of only 0.05 Felton units per cubic centimeter of serum.

Recovery without Demonstrable Antibody

Recovery without demonstrable protection during or immediately after the fall in temperature was noted in two of ten cases by Dochez (3). One of these (Experiment 9) showed protective substance 16 days after crisis, on the 22nd day of the disease. Clough (4) failed to demonstrate protective power after crisis or lysis in three of twelve cases, but in these three only one specimen of serum was tested. One (Case 9) of Baldwin and Rhoades' cases (5) had a crisis on the 15th day without protective substance in the serum, but later developed a fatal bacteriemia. Two of our seventeen untreated Type I cases had no demonstrable antibody at or near the time of crisis. In one of these, with crisis on the 8th day, the blood was negative when tested on the 8th, 9th, 10th, 12th, 14th and 20th days and was not tested thereafter. In the other, with crisis on the 6th day, the blood was negative on the 4th, 5th, 6th, 8th, 11th and 17th days but showed protection on the 25th, 32nd and 39th days after onset. A third Type I case, which received 60,000 units of Felton's Type I antibody near the time of his crisis on the 3rd day, showed no protection on the 3rd day (before serum), the irregular survival of only one of fourteen mice on the 5th day, and no protection on the 6th, 8th or 11th days, but by the 29th day after onset showed a relatively high degree of protection. The late appearance of protection in the last two cases is an assurance that the disease was due to a Type I infection. These three cases illustrate the importance of frequent repetition of tests to avoid erroneous negative results.

The significance of the absence of protective substance at the time of recovery and its appearance later is not clear. It is possible that mouse protection tests measure only an excess of antibody over the amount needed for recovery. It is also possible, in view of Goodner's (8) and Tillett's (9) inability to protect mice with immune rabbit serum that mouse tests as an index of available antibody are not wholly reliable and that a part of the human antibodies are undemonstrable with an alien species. Another explanation involves the presence in the blood serum of an antagonistic substance, sufficient in amount to inhibit mouse protection during the course of the infection and its disappearance after recovery.

One of our cases came to the hospital because of empyema following pneumonia and did not improve after surgical drainage. On the 83rd day of his disease the blood culture was positive for Type I pneumococcus in spite of the presence of mouse protection against 100,000 lethal doses. No specific treatment had been given. Autopsy 3 days later showed a Type I pneumococcus endocarditis.

The second case was observed throughout his disease* and was given no specific treatment. The blood was sterile on the 2nd day but from the 8th until death on

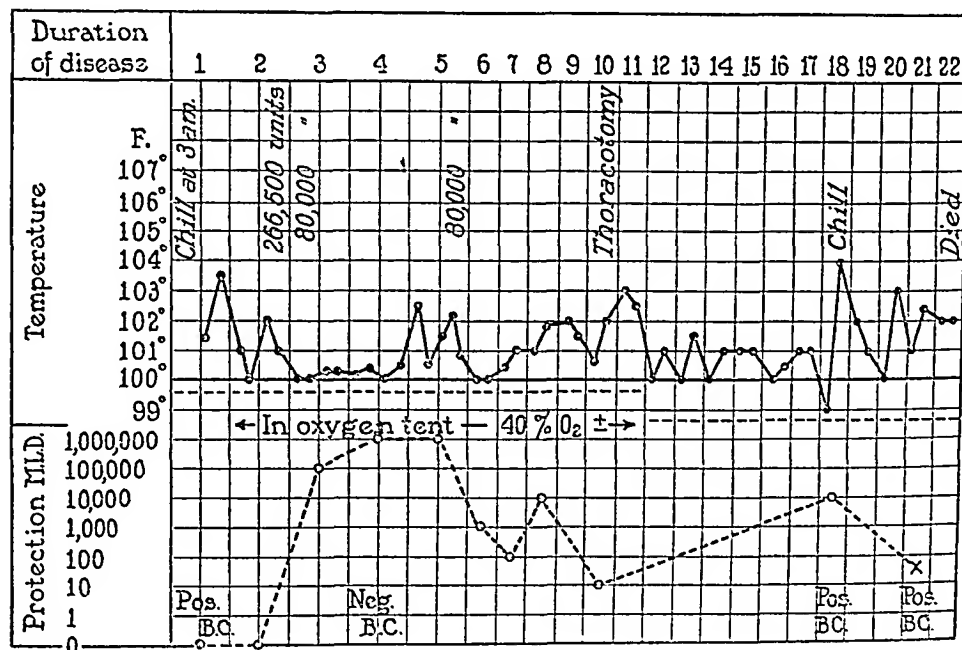


CHART 2. The presence of antibody with septicemia.

A severe case, showing the effect of treatment with antibody on the blood culture and protective substances early in the disease. After the antibody was discontinued the patient developed septicemia in spite of protective power in the blood.

The "normal" base line drops from 99.6° to 98.6°F. on the 12th day when mouth temperatures were substituted for rectal readings. o---o = amount of protection. X = irregular mouse survivals. B. C. = blood culture.

the 23rd day showed Type I pneumococci in each of nine cultures. No protective substance was demonstrated from the 10th to the 13th day by three tests, but on the 17th and 21st days, when blood culture showed a few organisms, his serum protected against 1,000 and 1,000,000 lethal doses respectively, and on the day of death there were numerous irregular mouse survivals. Thoracentesis on the 11th and 12th days produced infected fluid in small amounts but none was obtain-

* At the Boston City Hospital.

against every dose of culture used, up to and including one million lethal doses. Of five fatal treated cases, three (2 Type I, 1 Type II) showed the same protective power within 1 day of death. Thus, in 14 out of 34 treated cases it was possible to demonstrate more protective substance in the serum than would have been expected in untreated cases at the time the tests were made.

The Bearing of Protection Tests on Dosage

In attempting to estimate appropriate dosage by mouse protection tests account must be taken of the usual spontaneous production of small amounts of antibody during the course of the disease. The problem is complicated by occasional recovery without evidence of any protective substance and the lack of any definite correlation between the amount of protection and the time of recovery or the apparent severity of the disease.

It would be valuable to know the approximate dosage necessary to produce a large protective balance in the blood stream. In nineteen instances (18 Type I, 1 Type II) where protection tests* followed specific intravenous therapy with less than 200,000 Felton units between the 3rd and 6th days there was protection in amounts not otherwise to be expected in only six (all Type I). Of eight cases (7 Type I, 1 Type II) treated between the 2nd and the 7th days with over 200,000 units only one (Type I) failed to show unusual protective power. In general it may be said that doses of less than 200,000 Felton units are likely to be inadequate for the production of an unusual amount of protection in the blood stream. It must be added, however, that we have found protection against a million lethal doses in the serum after doses as small as 25,000 Felton units and yet this amount of protection has failed to appear after as much as 400,000 units.

The Presence of Antibody with Septicemia

In Baldwin and Rhoades (5) experience, bacteriemia and protective substances occurred simultaneously in the blood in only one instance in a total of 45 specimens of blood. We have been able to demonstrate protective substances late in the disease in blood from which pneumococci were cultivated in three instances.

* These tests were done on blood taken not less than 8 hours after treatment.

6. Protective substance in the blood and pneumococcic septicemia may occur simultaneously.

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able on the 18th day and there were no other signs of localization of the infection. Autopsy was refused.

In a third case (Chart 2), also Type I, there were extensive areas of consolidation involving all but the right middle lobe. In spite of early and intensive treatment a right-sided empyema developed. Respiratory exchange was maintained by the use of an oxygen tent and he did well until the 18th day when he had a chill and showed a positive Type I blood culture with protection in the blood serum against 10,000 lethal doses 13 days after his last treatment with antibody. There were no signs of extension of the lung involvement, but a rough systolic murmur and a pericardial friction rub persisted until death. No autopsy was granted.

These first two cases demonstrate that the formation of some protective substances by the patient himself is no assurance against further progress of the infection to a fatal termination. Although in the third case intensive specific therapy was used, the lapse of 13 days between the last dose of antibody and the time the final serum sample was taken makes it questionable whether the protective substances were due to the treatment or not.

CONCLUSIONS

1. Though in general in pneumococcus pneumonia the appearance of protective substance coincides rather sharply with the fall in the temperature, antibody may appear spontaneously in the blood serum as early as the 3rd or 4th day and crisis and recovery may be delayed until the 6th to the 10th day.
2. Recovery at times occurs without demonstrable protective substance in the blood in patients who later develop protection.
3. The amount of antibody developed in the course of pneumococcus pneumonia is small and in the majority of cases tested was insufficient to protect against more than 100 lethal doses of homologous pneumococci and never against more than 10,000 lethal doses.
4. Treatment with Felton's antibody late in the course of the disease materially increases the amount of protective substances in the blood. A high degree of protection may be established by treatment in fatal cases. After the 3rd day doses of more than 200,000 Felton units are usually necessary to produce a greater degree of protection than might otherwise be expected.
5. The formation of protective substances by the patient himself is not an assurance against progress of the infection to a fatal termination.

Febrile Reaction upon Reinoculation in Recovered Monkeys

In the first series of experiments three recovered monkeys (52, 36 and 40) were reinoculated by cerebral injection with the same strain of virus* (1 cc. of a 10 per cent cord emulsion); one normal monkey (B2) being infected at the same time for purpose of control. All three convalescent monkeys had recovered from a moderately severe attack of poliomyelitis 5 to 18 months previously, and still showed at the time of the experiment definite residual paralysis of the legs or arms. Needless to say, none of the recovered monkeys gave any evidence of a renewed attack of the disease during a period of observation of 6 weeks, while the control animal came down with typical poliomyelitis on the 9th day after inoculation. The control animal, in accordance with the observations of Kramer, Hendrie and Aycock, exhibited the characteristic febrile reaction the day before the clinical onset of the disease. The temperature curves† of the three recovered monkeys, on the other hand, showed a fairly well marked rise 24 hours after inoculation, followed by a rather sharp subsequent drop before normal values were reattained. (Graphs 1 and 2.)

In order to evaluate the significance of the transitory initial rise of temperature in the recovered monkeys, particularly so as to avoid confusion with the somewhat similar early traumatic reaction in the control, we have, in a second series of experiments, analyzed the febrile reaction during the first 48 hours after inoculation by taking several daily readings at frequent intervals. Thus, another group of four recovered monkeys was reinoculated by intracerebral injection, the experiment being controlled by simultaneous inoculation of two normal monkeys (B9 and B8). In two of the recovered monkeys (A18 and A91) the onset of the previous infection dated back 3 and 6 months, respectively, while the remaining two convalescents (A70 and A95) had barely recovered from a very recent attack of the disease

* The virus used in this work was a strain isolated by Dr. Aycock.

† The temperature curves were compiled from daily rectal measurements taken during the incubation period. Before taking the temperature, the monkeys should be subjected to as little physical exertion as possible. The same certified thermometer was used throughout this work. All readings are noon temperatures (3 minutes) unless otherwise indicated. Whenever an animal died, a careful autopsy was made to rule out any possible enteric infection or tuberculosis.

AN ACCELERATED FEBRILE REACTION IN MONKEYS UPON REINOCULATION WITH POLIOMYELITIS VIRUS*

By CLAUD W. JUNGBLUT, M.D.

(From the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York)

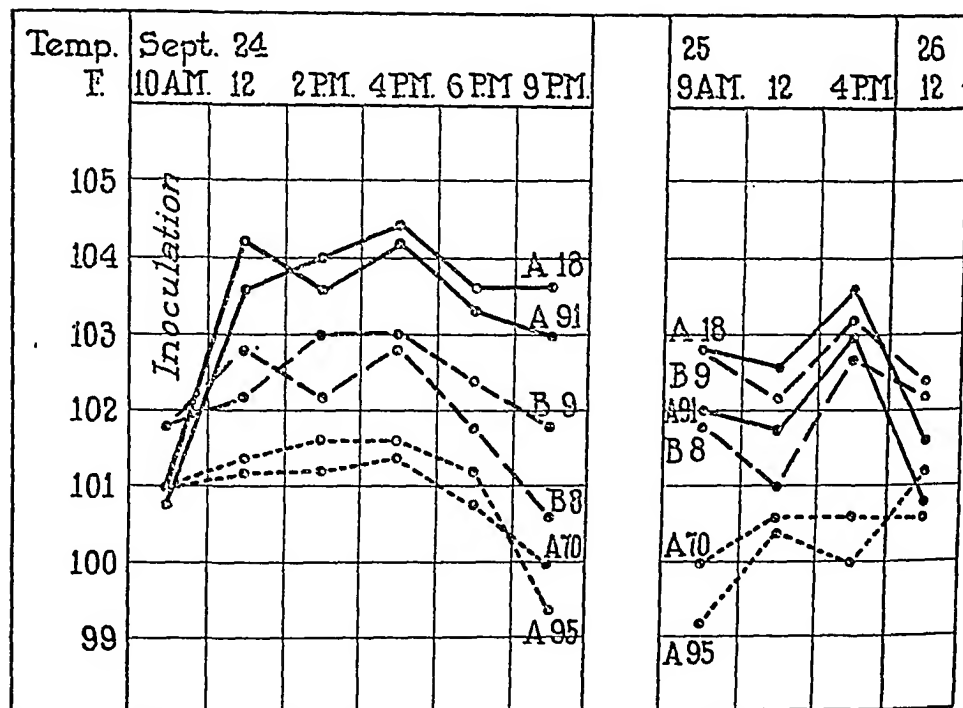
(Received for publication, October 30, 1930)

The course of poliomyelitic infection in monkeys during the incubation period has recently been made the object of careful study by Kramer, Hendrie and Aycock (1). As the result of these investigations the authors have described a regular and distinct rise in temperature during the preparalytic stage, occurring as a rule 1 or 2 days before the onset of the first clinical symptoms of the disease. This fundamental observation, which incidentally agrees with a similar report of Fairbrother and Hurst (2) is of particular interest inasmuch as it provides a deeper insight into that latent phase of the infection during which the virus grows in the tissues preparatory to the production of lesions.

In the following study we were primarily concerned with the febrile response in monkeys, which had previously been in contact with poliomyelitis virus, upon reinoculation with the same virus. Reasoning by analogy with other infectious processes, we should expect that previous contact with the antigen would leave the reacting susceptible tissue cells in a state of altered receptivity, one phase of which might conveniently be detected through observation of the body temperature. In order to study the various aspects of this problem we have traced the temperature curves, after reinoculation, in monkeys which were in various stages of convalescence from a preceding poliomyelitic infection and in monkeys which had received a number of injections of live virus by various routes of administration in an attempt at active immunization (3).

* Under a grant from the International Committee for the study of infantile paralysis, whose work is being financed by Jeremiah Milbank.

"old convalescents" responded within the first 2 hours after inoculation with a high and sustained rise of the temperature, the body temperature of the two "recent convalescents" showed no more fluctuation than is common for the daily physiological oscillation. In contrast herewith, the temperature in the two controls reached intermediate values such as have been reported before by Kramer, Hendrie and Aycock.

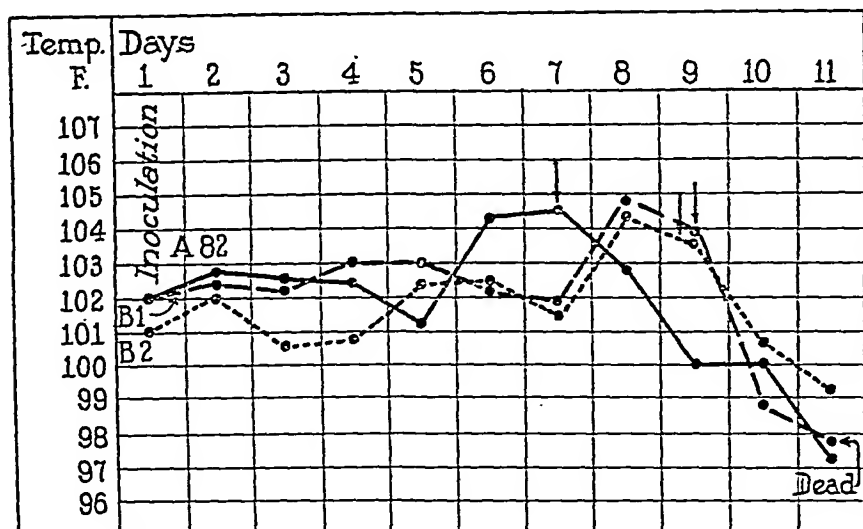


GRAPH 3. Comparison of febrile reaction during the first 48 hours after reinoculation in recovered monkeys with febrile reaction of primary infection.

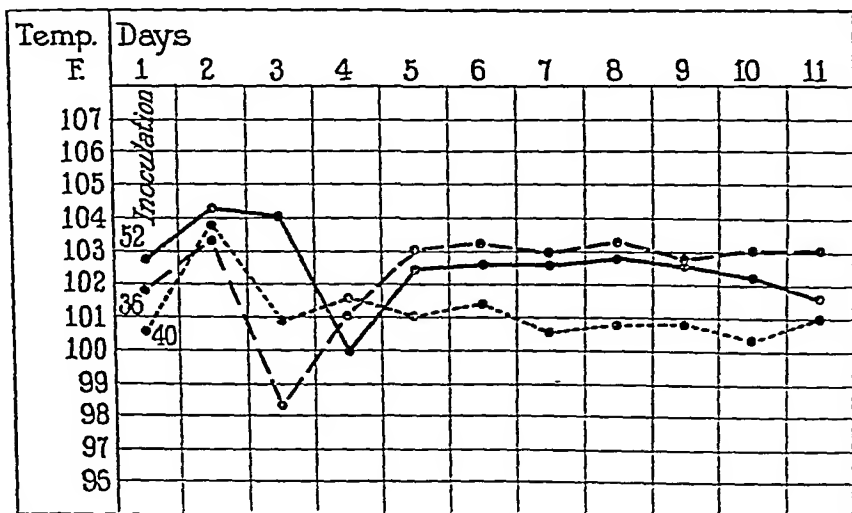
- Old convalescents (A18, A91).
 ——— Normal controls (B9, B8).
 - - - - - Recent convalescents (A70, A95).

In the further course of this work we were interested in investigating whether killed virus was capable of eliciting a febrile response in recovered monkeys, comparable to that following reinoculation with live virus. At the same time it became important to ascertain how the reaction would proceed; if the antigen was introduced by the subcutaneous route, a method which circumvented the acute inflammatory reaction incidental to intracerebral inoculation. Accordingly,

(2 to 3 weeks before). The temperature curves (Graph 3) demonstrate three different types of febrile reaction in those six animals, each type characteristic for each group of monkeys. While both of the



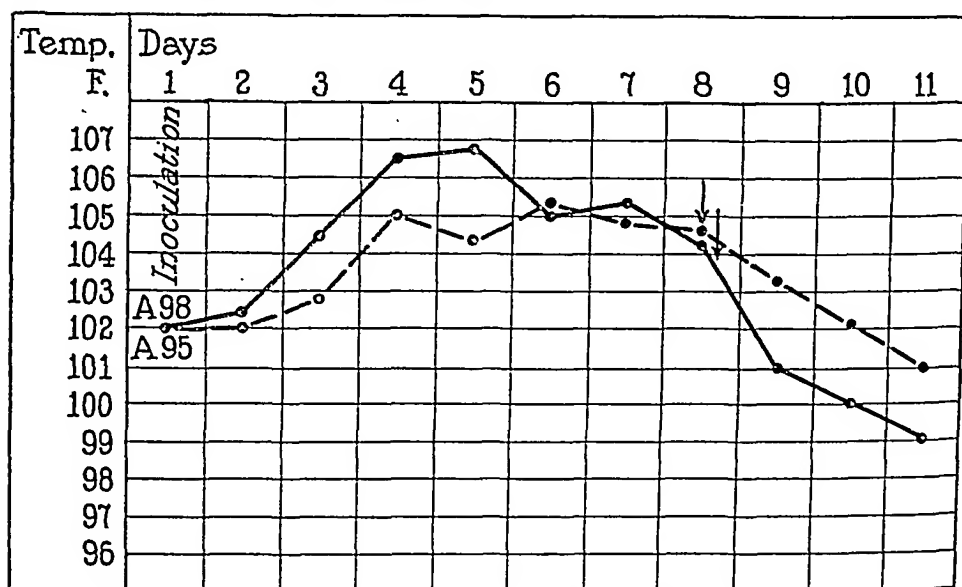
GRAPH 1. Primary infection in three control monkeys (A82, B1 and B2). The arrows indicate the onset of symptoms.



GRAPH 2. Reinoculation of three recovered monkeys (52, 36 and 40).

subcutaneous injection of killed virus in two of the recovered monkeys (86, 76), while the temperature of the two control animals (6, B23) remained within physiological limits during the period of observation (72 hours).

In repeating the fundamental experiments described above, either with the same arrangement or under slightly differing conditions, essentially identical results were obtained in each instance, although the intensity of the febrile reaction as to promptness and magnitude has naturally shown some variation from animal to animal. It seems only logical to assume that recovery from the disease, even at the same fixed time after infection, leaves no two individuals in exactly the same state of immunity.

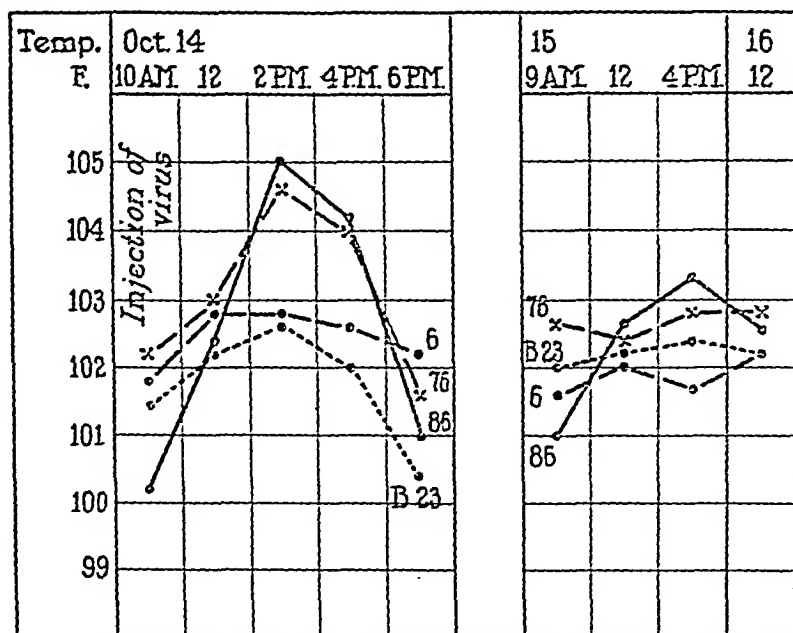


GRAPH 5. Inoculation of two sensitized monkeys (A98 and A95). The arrows indicate the onset of symptoms.

Febrile Reaction upon Inoculation in Sensitized Monkeys

Inasmuch as the previous work had shown that recovery from poliomyelitic infection is associated with an altered response of the convalescent animal towards reintroduction of the same antigen, it became of interest to ascertain whether a similar allergic reaction could be demonstrated upon reinoculation of monkeys, in which artificial immunization by treatment with subinfective doses of virus had been attempted. To study this phase of the problem, six monkeys

two recovered monkeys, both of which gave a history of a mild attack of poliomyelitis several months before, were injected with killed virus (10 per cent virus cord suspension, heated for $\frac{1}{2}$ hour at $65^{\circ}\text{C}.$), the one animal (86) receiving 1 cc. of the supernatant intracerebrally, the other (76) 1.5 cc. by subcutaneous injection. For purpose of control a third recovered monkey (6) was inoculated intracerebrally



GRAPH 4. Febrile reaction during the first 48 hours after injection of killed virus in recovered monkeys.

- Recovered monkey injected intracerebrally with killed virus (86).
- x-x- Recovered monkey injected subcutaneously with killed virus (76).
- Recovered monkey injected intracerebrally with normal monkey cord emulsion (6).
- Normal monkey injected intracerebrally with live virus (B 23).

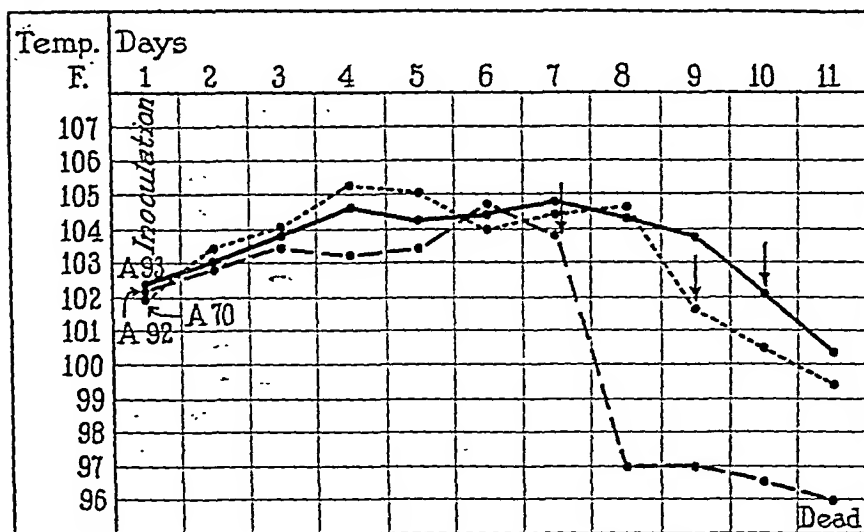
with 1 cc. of the supernatant of a 10 per cent suspension of normal monkey cord, a fourth normal monkey (B23), infected with live virus intracerebrally, completing the experimental series. The temperature curves of the four animals, as recorded in Graph 4, demonstrate again an immediate and sharp rise of the temperature after intracerebral injection and a less prompt though distinct febrile reaction following

over a period of 6 weeks (3). Five of these (A70, A92, A93, A95, A98), after a free interval of approximately one month, were inoculated by cerebral inoculation with the same strain of virus, together with one normal control (A82), the sixth immunized animal (A94) receiving an intracerebral injection of normal monkey cord instead. All monkeys inoculated with the virus came down promptly, from 7 to 10 days later, with typical poliomyelitis, the severity of the disease in the treated group hardly differing from that of the control. Inspection of the temperature curves, however (Graphs 1, 5 and 6), reveals an entirely different response of the treated animals to inoculation as compared with the febrile reaction of the primary infection in the control monkey. While the latter went through the typical febrile reaction,—the temperature remaining practically normal during the entire incubation period except for an abrupt rise 24 or 48 hours before the development of objective clinical symptoms,—the temperature curves in four of the five treated animals showed a precocious elevation on the 3rd or 4th day after inoculation with a high plateau during the remainder of the incubation period. The injection of normal cord in the sixth treated animal did not affect the normal body temperature (Graph 7).

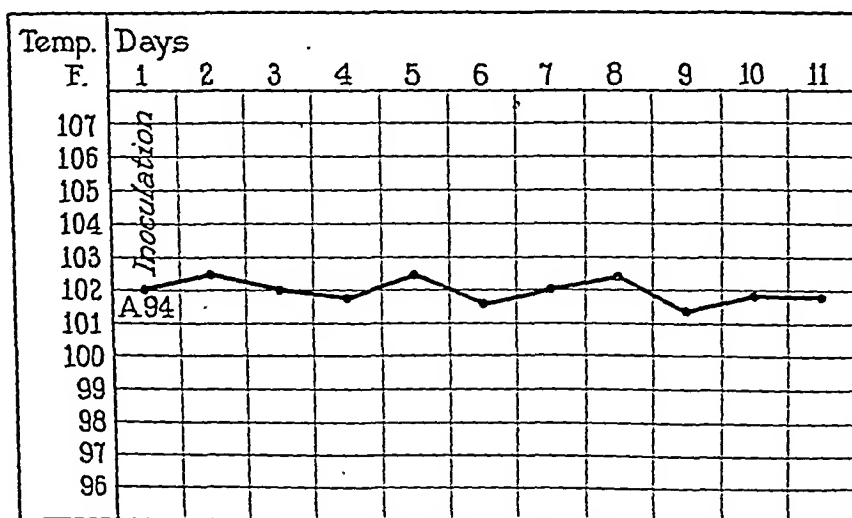
DISCUSSION

The observations recorded in this paper are of interest inasmuch as they remind one forcibly of the accelerated febrile reactions observed with allergic phenomena in general, such as the pyrogenic reaction of Friedberger's in classical anaphylaxis, the syndrome of serum sickness, the tuberculin reaction and, finally, vaccinal allergy (v. Pirquet, Force and others). In each of the instances quoted antigenic substances, which in the normal animal produce either no effect at all or lead to a train of pathological symptoms only after a definite and fixed incubation period, cause an accelerated reaction upon reintroduction into a specifically sensitized individual. The obvious parallelism between such allergic reactions and the accelerated febrile response to poliomyelitic reinoculation in monkeys which had previously been in contact with the virus, suggests that sensitization in poliomyelitis may be of frequent occurrence. As a matter of fact, several authors have entertained this viewpoint before, without however

were available, all of which had received a number of injections of live virus by different routes (subcutaneous, intraperitoneal, intrarectal)



GRAPH 6. Inoculation of three sensitized monkeys (A93, A92 and A70). The arrows indicate the onset of symptoms.



GRAPH 7. Temperature curve of sensitized monkey after intracerebral injection of normal monkey cord (A94).

If sensitization to the virus occurs as readily as our data would seem to indicate, the observations made in this paper may also furnish a possible explanation for the difficulties commonly encountered in obtaining protection with any degree of regularity against the disease in monkeys by the ordinary methods of active immunization (3). Even intracerebral inoculation with a subinfective dose of virus, according to Amoss (6), instead of conveying immunity to the monkey, tends to render the animal more susceptible to a repeated inoculation. Of particular interest in this connection is the fact that the highest immunity index was obtained by Ayccock and Kagan (7) with prolonged intradermal immunization, a procedure which carries the essential characteristics of a desensitization process. But even this method rarely insures protection against more than one subsequent test infection, the immunized animals frequently succumbing to repeated inoculation. This observation, which otherwise is wholly incompatible with the conception of solid immunity in virus diseases, may readily be explained on an allergic basis.

The described accelerated febrile reaction appears to be, at present, the only demonstrable indication for the participation of allergic factors in the immunity against poliomyelitis, since we have been repeatedly unsuccessful in eliciting any local hyperergic reaction in convalescent or immunized monkeys in which the virus was either injected intradermally or instilled into the conjunctival sac.

SUMMARY AND CONCLUSIONS

1. Primary poliomyelitic infection in the monkey, as a rule, is characterized by no significant increase in the body temperature during the incubation period until 48 or 24 hours before the onset of clinical symptoms, when a critical rise of the temperature occurs.

2. The temperature curve of recovered monkeys on intracerebral reinoculation shows an almost immediate and marked febrile reaction during the first 24 or 48 hours after inoculation. A similar accelerated febrile reaction may be obtained in recovered animals after subcutaneous injection of killed virus. In case the previous infection is of very recent date, reinoculation may lead to no demonstrable reaction whatsoever.

being able to adduce convincing experimental evidence for its support (Roemer (4), Shaughnessy, Harmon and Gordon (5)).

There can hardly be any doubt but that the accelerated febrile reactions observed by us under various circumstances were due to specific factors. This is proven not only by the absence of any reaction after intracerebral injection of normal monkey cord into recovered or sensitized monkeys, but more particularly supported by the fact that the type of sensitization apparently is determined by special conditions of tissue susceptibility. Thus we find monkeys shortly after an attack of poliomyelitis completely anergic to reinoculation with virus. As the interval increases, the febrile response of the recovered animal is an almost immediate one and of marked intensity, though of short duration. In either instance the animals enjoy complete protection against another attack of the disease. On the other hand, monkeys which have received a number of subcutaneous or intraperitoneal injections of live virus respond, on the average, 3 or 4 days after cerebral inoculation with a sharp and prolonged rise of the temperature, in the majority of the cases eventually succumbing to the infection in spite of the attempted immunization. In primary infection, finally, the disease runs practically an afebrile course during the entire incubation period,—except for a slight, transitory traumatic reaction on the day or the day after the infection,—until 24 or 48 hours before the onset of clinical symptoms, when a critical rise of the temperature occurs.* Thus, there appears to exist a close correlation between the different degrees of increased or decreased susceptibility and the type of febrile reaction. It is quite possible, that the allergic fever reaction could be used as an index for susceptibility in the human, provided that current interpretation of the insusceptibility of the older age groups on the basis of a latent immunization by repeated contact with subinfective doses of virus is in accordance with the actual facts. The possibility of using killed virus subcutaneously for this purpose would render such a test practicable. Further experiments will have to elucidate the relation between allergy and circulating viruscidal antibodies.

* While in most cases the temperature curves of primary infection have conformed with this type of febrile reaction, we have occasionally observed a more gradual development of the fever during the latter third of the incubation period, particularly in the milder and subacute cases.

3. Monkeys, which have received a number of parenteral injections of live virus, respond to intracerebral infection with a precocious and prolonged febrile reaction on the 3rd or 4th day after infection which may last until the onset of symptoms.

4. The altered response to reinoculation of monkeys which have previously been in contact with the virus, suggests a close analogy with the accelerated reactions observed in allergic phenomena.

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The earlier literature on the subject has been reviewed elsewhere (1, 2). With the demonstration that the dissemination of bacteria was retarded by an inflammatory reaction (5, 6, 7) and with the subsequent studies on the fixation of a dye, iron, and foreign protein at the site of inflammation, it became of interest to study the mechanism of fixation by the inflammatory reaction. It had been shown (2) that the accumulation of a dye in an inflamed area was doubtless connected with increased capillary permeability. In this paper an attempt is made to study the mechanism involved in the fixation of foreign substances by the inflammatory reaction.

The leucocytes are probably not a very significant factor in the mechanism of fixation for two reasons. Histologically no definite evidence could be obtained of phagocytosed particles in the leucocytes of the inflamed area at a time when fixation of foreign substances was already demonstrable by examining the tributary lymphatics. In the second place, fixation of trypan blue at the site of inflammation was shown to occur as early as 30 minutes after the injection of the inflammatory irritant (1). The occurrence of fixation at this early stage of the inflammatory reaction when there are as yet relatively few leucocytes present seems to point toward some other factor responsible for fixation.

Schade and Menschel (8) have found that in inflamed areas, especially those with suppuration, the accumulation of products of tissue disintegration may become so great that the osmotic pressure is raised to as high as eleven atmospheres, with a simultaneous marked increase in hydrogen ion concentration. They believe that inflammatory edema has primarily an osmotic origin. For this reason it seems reasonable enough to suppose that the increase in osmotic pressure at the site of inflammation might be a factor in influencing the flow of fluids and perhaps indirectly any contained substances.

A factor which might explain fixation is mechanical obstruction. It is conceivable that a network of fibrin and thrombosed lymphatics at the site of inflammation might arrest the passage of particulate material injected into such an inflamed area. The dissemination of fluids would probably also be retarded by mechanical obstruction of this kind, though probably not as effectively as solid particles which would be more readily caught in a fibrinous network.

STUDIES ON INFLAMMATION

V. THE MECHANISM OF FIXATION BY THE INFLAMMATORY REACTION

By VALY MENKIN,¹ M.D.

(From The Henry Phipps Institute, University of Pennsylvania, Philadelphia, and the Department of Pathology, Harvard Medical School, Boston)

PLATE 7

(Received for publication, October 31, 1930)

In previous communications it was shown (1) that trypan blue injected into the circulating blood rapidly enters the site of inflammation and is fixed there, so that the tissues are deeply stained. Furthermore trypan blue injected directly into the site of inflammation in the subcutaneous tissue or in the peritoneal cavity is fixed in the inflamed area and fails to reach the regional lymphatic nodes. Subsequent studies showed that the rapid accumulation of dye in an inflamed area is associated with increased capillary permeability (2). These studies were then extended and it was found that colloidal iron or ferric chloride injected directly into an inflamed area was fixed *in situ* by the inflammatory process, and that ferric chloride injected intravenously rapidly entered inflamed cutaneous areas, where its presence was identified by both qualitative and quantitative determinations (3). Further studies demonstrated that a foreign protein, as *e.g.* horse serum, injected into an inflamed peritoneal cavity penetrated into the blood stream less rapidly than when introduced into the normal cavity (4). When the foreign protein was injected into a cutaneous inflammatory area it was held *in situ* for a longer period than when injected into an inflamed peritoneal cavity. It was also found that foreign protein introduced into the circulating blood stream accumulated in an inflamed area, where it was present in greater concentration than in normal tissue.

¹ A part of this study was performed under a Fellowship in Medicine of the National Research Council.

Having shown the direct effect of exudate on an iron salt, experiments were then undertaken to determine the influence of the inflammatory exudate and of the blood serum on foreign protein such as horse serum.

The inflammatory exudate was obtained as described above. After centrifugalizing the exudate a piece of tissue, either kidney or lymph node, was added to the supernatant fluid to bring about prompt and firm coagulation. The coagulated material was again centrifugalized and the supernatant fluid used for the experiments. 0.5 cc. of varying dilutions of horse serum was added to 0.5 cc. of exudate or to 0.5 cc. of blood serum. For each experiment two control test tubes were set up, one containing 0.5 cc. of the exudate and the other 0.5 cc. of blood serum. Both were brought up to 1 cc. with saline. The tubes were then placed in a water bath at 37°C. for from 15 to 25 minutes.

The control tubes and those containing blood serum showed no trace of coagulation on the addition of horse serum, whereas when the latter was mixed with inflammatory exudate marked coagulation resulted in all cases.

Experiments similar to the above were performed with trypan blue, but in no instance could it be shown that either exudate or blood serum had any precipitating or coagulating effect on this dye. Yet trypan blue is fixed *in situ* by the inflammatory process. For this reason it is believed that fixation *per se* involves primarily a different mechanism than precipitation. However, since it has been found that iron is more effectively fixed than trypan blue in an inflamed peritoneal cavity (1, 3) it is possible that precipitation of the ferric salt plays some part in accentuating the effect of the fixation mechanism. Precipitation or coagulation of a foreign substance when injected in an inflamed area may be a secondary factor in preventing its rapid dissemination from the site of inflammation.

Histological Studies of Area of Inflammation

Sections were made of the inflamed tissue of rabbits in experiments in which either trypan blue or ferric chloride had been shown to be fixed *in situ* by the inflammatory process. There is as a rule a central area of dense leucocytic infiltration. The intensity of the inflammatory reaction in the immediate neighborhood of veins and arteries is noteworthy. It is to be recalled (1) that when the dye was injected

In this connection it is interesting to note that some years ago Opie (9) showed that when cantharidin is administered intramuscularly thoracic duct lymph flow is at first diminished but later may be increased. The decrease of lymph flow was accompanied by acute edema of the liver and gall bladder. This edema was due to plugging by fibrin of the afferent lymphatics and the sinuses of lymph nodes which drain these organs. The observations of Adami (10) are also significant in this connection:

"Even when inflammation (as in pericarditis) affects the whole extent of a serous cavity, the layer of fibrin acts as a protective coat closing the lymphatic 'stomata' hindering the free absorption of the morbid material by the lymph and blood vessels, and filtering bacteria out of such fluid as does find its way through to the tissues beneath."

In the endeavor to throw some light upon the mechanism involved in fixation, a series of experiments was undertaken to determine whether the inflammatory exudate in itself possessed some property which might facilitate the fixing of foreign substances in the inflamed area.

The Effect of Adding Ferric Chloride, Horse Serum, or Trypan Blue to the Inflammatory Exudate and to Blood Serum

The inflammatory exudate was obtained by injecting 0.5 cc. of 10 per cent croton oil in olive oil into the peritoneal cavity of rabbits. 24 to 72 hours later the exudate was removed and centrifugalized. A sample of blood was also removed from the heart and likewise centrifugalized. The tests were made by the addition of 0.2 cc. of varying dilutions of a 0.25 per cent ferric chloride solution to 0.3 cc. of either exudate or blood serum. The final volume was brought up to 1 cc. with saline (0.9 per cent).

Addition of ferric chloride to the inflammatory exudate gave rise in each case to a heavy precipitate. Addition of the undiluted iron salt to blood serum produced a precipitate which on slight shaking immediately redissolved. At higher dilutions of ferric chloride solution no precipitation occurred when it was added to blood serum. That the precipitate caused by undiluted 0.25 per cent ferric chloride solution and blood serum is transient and redissolves on slight shaking is of some interest in explaining the possibility of injecting the ferric salt intravenously (3, 11) without fatal embolic effect on the animal.

In view of the work of numerous investigators it is probable that the precipitate formed by the addition of ferric chloride to the inflammatory exudate is a ferric proteinate (12).

The results are shown in Table I. It is seen that the dye failed to penetrate into the inflamed area when injected at its periphery, whereas it disseminated readily into the control area. The area of inflammation usually appeared as a definite round spot free from blue coloration. At the conclusion of two of the experiments (1 and 2) 10 cc. of 1 per cent trypan blue were injected into the circulating blood stream of each animal. It is interesting to note that within a short interval of time the inflamed area into which no trypan blue had penetrated when injected at its periphery was now distinctly stained by the dye.

TABLE I

The Penetration of Trypan Blue into an Inflamed Cutaneous Area When the Dye Is Injected at Its Periphery

Experiment	Interval between injection of irritant and that of dye	Total duration of inflammation	Penetration of dye into inflamed area	Penetration of dye into normal area
	<i>hrs.:min.</i>	<i>hrs.:min.</i>		
1*	0:40	4:00	0	+
2*	1:00	4:30	0	++
3**	18:00	19:00	0	++
4	19:45	21:20	0	++
5**	21:45	25:30	0	+
6	22:05	25:40	0	++

* The inflammatory reaction was caused by concentrated broth.

** The site of inflammation was located in the foreleg.

Similar results obtained on the frog will be reported in a separate paper.

DISCUSSION AND CONCLUSIONS

Microscopic studies show the presence of a network of fibrin within the tissues and numerous thrombosed lymphatics at the site of inflammation. Precipitated iron compounds, possibly coagulated horse serum, or particulate matter caught in this fibrinous reticulum will disseminate less readily than trypan blue from the site of inflammation.

Trypan blue injected at the periphery of an inflamed area fails to enter the site of inflammation. This failure of penetration is caused

intravenously it would not always penetrate into the central zone of the inflamed area. This is evidently due to thrombosis of the small vessels for sections of such areas reveal some thrombosed vessels with acute inflammatory changes in the surrounding tissue.

Histologically there is little evidence of phagocytosed particles of trypan blue or of iron within the leucocytes at a time when retention of these substances at the site of inflammation is clearly demonstrable.

It is of interest to note the meshwork of fibrin which is found usually at the periphery of the zone of dense infiltration (Fig. 1). In the same region careful study reveals many lymphatic vessels which are thrombosed. Fig. 2 shows very clearly an occluded lymphatic vessel. The thrombus is characterized by numerous leucocytes within a delicate fibrinous reticulum. The fact that there are many occluded lymphatics and a dense network of fibrinous strands within tissues that are distended with edema at the site of inflammation supports the view that foreign substances, especially solid particles, such as precipitated iron salts, can disseminate only with difficulty from the inflamed area through the regional lymphatic vessels.

The Failure of Trypan Blue to Penetrate into an Inflamed Area When the Dye Is Injected at Its Periphery

If, as described above, the thrombosed lymphatics and the network of fibrin in an acutely inflamed area are instrumental in preventing mechanically the free passage of substances from the site of inflammation, it follows that for the same reason similar substances injected at the periphery of the inflamed area should fail to enter it. To test this hypothesis, the following experiments were conducted at the suggestion of Professor Eugene L. Opie.

An inflammatory reaction was induced by the injection of 0.4 cc. of a saline suspension of *Staphylococcus aureus* into either the skin of the abdomen or into the subcutaneous tissue of the foreleg of a rabbit 2 or 3 cm. from the shoulder joint. After a variable interval of time about 1 cc. of 1 per cent trypan blue was injected into four to six areas of the skin immediately adjacent to the site of inflammation. Thus the inflamed area became circumscribed by a colored band of blue. In a normal skin area of the abdomen of the same size as the inflamed area or in the normal foreleg similar injections of dye were made to serve as controls. In two experiments 0.5 to 0.6 cc. of concentrated broth was used as the inflammatory irritant. Several hours later both inflamed and normal areas were examined for the presence of dye.



by the occlusion of lymphatic vessels and by the presence of a fine network of fibrin in the tissue spaces of the inflamed area.

Fixation of foreign substances by the inflammatory reaction is therefore primarily due to mechanical obstruction caused by a network of fibrin and by thrombosed lymphatics at the site of inflammation.

There is another phase of the problem which still requires more accurate information. This concerns the relation between exudation from blood vessels and changes in flow of lymph from the inflamed area. Further experiments are being conducted to investigate this question.

The reaction of fixation which occurs extremely early in the inflammatory process circumscribes the irritating substance and allows a definite period of time for the leucocytes to assemble for the purpose of phagocytosis.

It is through a delicate regulating mechanism of this kind that, to use the expression of Opie (7), "the vital organs are protected at the expense of local injury."

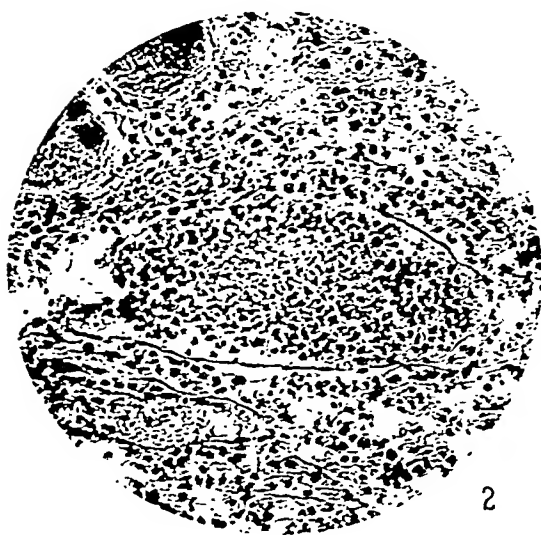
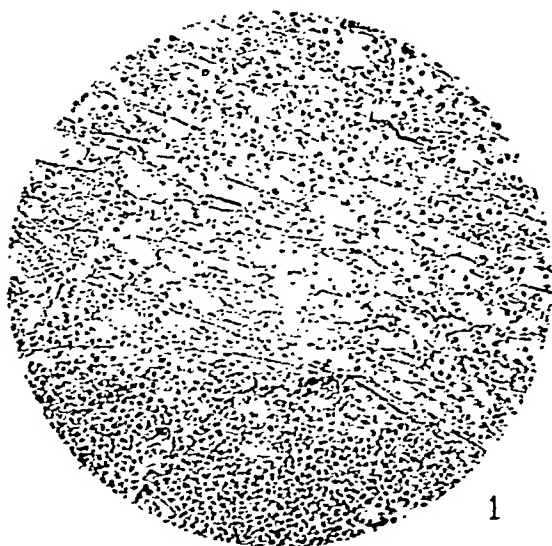
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EXPLANATION OF PLATE 7

FIG. 1. Site of inflammation showing network of fibrin in the subcutaneous tissue of rabbit. Trypan blue was injected directly into this area and was shown to be fixed *in situ*. Low power magnification.

FIG. 2. Site of inflammation. Same section as in Fig. 1 but in a different field. A lymphatic vessel occluded by a large thrombus. High dry magnification.



In the control animal the dye had distinctly diffused throughout the body. The buccal cavity, tongue, abdominal wall, and both thighs were distinctly blue. The visceral organs and the heart were also stained. Examination of the blood obtained from the region of the sinus venosus clearly showed the presence of the dye. The experimental frogs, on the other hand, while characterized by intense staining at the site of the inflammation, showed in general no trace of the dye in the buccal cavity, tongue, and abdominal wall. The normal thigh, the visceral organs, the heart, and blood were as a rule devoid of any trypan blue. Only animals that displayed active muscular behavior throughout the period of the experiment were used. The results of all the experiments appear in Table I.

TABLE I

Dissemination of Trypan Blue from the Site of Inflammation in Frogs

Experiment	Interval between injection of irritant and that of dye	Total duration of inflammation	Presence of dye in buccal cavity of animals with inflamed thigh	Presence of dye in buccal cavity of animals with normal thigh
	<i>hrs.:min.</i>	<i>hrs.:min.</i>		
1	0:47	1:52	0	+++
2	4:25	9:30	0	++
3	6:25	11:42	0	+++
4	6:35	12:05	Trace	+++
5	7:30	10:00	0	+++
6	20:30	22:30	0	++
7	24:30	25:40	0	++

For the sake of convenience the tabulation includes a comparison of the intensity of dye only for the buccal cavity of both normal frogs and of frogs in which there is acute inflammation in the thigh. It is clear from the results that in frogs as in rabbits, trypan blue is also fixed *in situ* by an inflammatory reaction.

In the following group of experiments the attempt was made to determine whether in frogs the dye injected into the circulating blood would accumulate rapidly at the site of inflammation.

Acute inflammation was induced in the thigh by means of croton oil as described in the preceding experiments. After an interval of time varying in different experiments from 35 minutes to about 17 hours the brain of the frog was pithed.

STUDIES ON INFLAMMATION

VI. FIXATION OF TRYPAN BLUE IN INFLAMED AREAS OF FROGS

By VALY MENKIN,¹ M.D.

(From The Henry Phipps Institute, University of Pennsylvania, Philadelphia, the Marine Biological Laboratory, Woods Hole, and the Department of Pathology, Harvard Medical School, Boston)

(Received for publication, October 31, 1930)

Heretofore all experiments on fixation of substances at the site of inflammation have been performed on rabbits (1, 2, 3, 4). However, it has been shown in a previous communication (5) that in the frog the rate of fall of concentration of a dye in the capillaries of an inflamed area was greater than in capillaries of a corresponding normal area. The difference represented a measure of increased capillary permeability with inflammation. In order to relate that study to the observations obtained in rabbits on the fixation of foreign substances at the site of inflammation, a series of experiments was undertaken to demonstrate fixation and accumulation of trypan blue in inflamed areas of frogs. On extending these experiments a certain amount of information was obtained which lent further support to the hypothesis that fixation is primarily the result of mechanical obstruction caused by a network of fibrin and thrombosed lymphatics at the site of inflammation (6).

0.1 to 0.2 cc. of 10 per cent croton oil in olive oil was injected into the large subcutaneous lymph space of the thigh of a medium or large sized bull-frog (*R. catesbiana*). After a period ranging from 45 minutes to 1 day distinct hemorrhagic signs became visible at the site of injection. 0.5 cc. of 1 per cent trypan blue was then injected directly into the area of inflammation. 0.5 cc. of the dye was also injected into the same lymph space in the thigh of a normal frog acting as control. After several hours both frogs were pithed and various organs examined for the presence of dye.

¹ A part of this study was performed under a Fellowship in Medicine of the National Research Council.

spectively. Sometimes the dye was also injected subcutaneously in the right thigh. In a normal frog, acting as control, similar injections of dye were made. Several hours later both frogs were pithed and the inflamed thigh as well as the corresponding thigh in the control animal were studied for the presence of the dye.

The results of the experiments appear in Table III. It is clear that whereas trypan blue always diffused into the normal thigh of the control animal, no dye penetrated into the inflamed area of the experimental frog. Often the dye would be seen bulging against the fibrous septum at the periphery of the inflamed thigh without actually penetrating into it. Microscopic section of the skin of the inflamed thigh

TABLE III

The Penetration of Trypan Blue into an Inflamed Area When the Dye Is Injected around It

Experiment	Interval between injection of irritant and that of dye	Total duration of inflammation	Penetration of dye into inflamed thigh of experimental animal	Penetration of dye into normal thigh of control animal
	<i>hrs.:min.</i>	<i>hrs.:min.</i>		
15	3:10	4:10	0	+
16	13:00	16:00	0	++
17	13:30	15:30	0	+
18	14:00	16:30	0	Trace to +
19	14:00	17:00	0	++
20	14:00	17:00	0	++
21	14:00	15:30	0	Trace
22	14:15	15:35	0	+
23	15:00	16:30	0	+
24	15:00	17:00	0 to trace	+

showed not only some capillaries packed with red cells and large numbers of extravasated cells in the tissue spaces with the occurrence of fibrin deposit, but also some thrombosed lymphatics. In a skin specimen removed from the inflamed area, direct examination under a binocular dissecting microscope revealed some lymphatic vessels that form part of the deep cutaneous network to be diffusely blue at the periphery of the inflamed area but on following one of these lymphatics as it entered the inflamed area it abruptly became colorless. The point in the vessel where the diffusion of the dye was blocked revealed a large clot occluding the lumen.

The heart was exposed and the pericardial sac slit open. 0.8 to 1 cc. of 1 per cent trypan blue was slowly injected into the ventricle. Within about a minute the inflamed thigh became intensely blue. The normal thigh at this time showed considerably less coloration, if any at all.

The results are shown in Table II. It is to be noted that when the inflammatory reaction was of very long duration and the skin was particularly congested, the dye did not penetrate into the inflamed skin. The capillaries in these experiments were found to be thrombosed and in complete stasis. If, however, the inflamed muscles of the thigh were incised in such experiments, a greater accumulation of the dye was observed in them than in the muscles of the normal thigh.

TABLE II
Accumulation of Trypan Blue at the Site of Inflammation in Frogs

Experiment	Duration of inflammation <i>hrs.:min.</i>	Presence of trypan blue in inflamed thigh	Presence of trypan blue in normal thigh
8			
9	0:37	+++	0
10	0:38	+++++	+
11	2:33	+++++	Trace
12	3:00	++	+
13	3:10	+++++	Trace to +
14	3:30	+++	0
	16:40	+	Trace

The Failure of Trypan Blue to Penetrate into an Inflamed Area When the Dye Is Injected at Its Periphery

In a previous study (6) it has been shown that trypan blue injected at the periphery of an inflamed area in rabbits failed to penetrate into it. This, as histological evidence pointed out, was due to the presence of a fine reticulum of fibrin and to thrombosed lymphatics at the site of inflammation. These findings are reproduced in the following experiments on frogs.

An acute inflammatory reaction was induced, usually in the left thigh of medium sized bull-frogs (*R. catesbiana*) by the injection of croton oil as described above. After a variable interval of time 0.1 to 0.2 cc. of 1 per cent trypan blue was injected in three subcutaneous lymph spaces as follows: into that of the left lower quadrant of the abdomen, in that over the dorsum of the left and right feet re-



CONCLUSIONS

Trypan blue injected into the circulating blood stream of frogs accumulates rapidly in an inflamed area.

When trypan blue is injected directly into the area of inflammation it is fixed *in situ* and fails to diffuse outward.

If the dye is injected at the periphery of an inflamed area it fails to enter the site of inflammation. This failure of penetration is caused by the occlusion of lymphatic vessels and the presence of a network of fibrin in the inflamed area.

These experiments furnish additional evidence that fixation of foreign substances by the inflammatory reaction is due to mechanical obstruction caused by a network of fibrin and by thrombosed lymphatics at the site of inflammation.

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was no gross evidence of generalized vaccinia; but histological studies were not made. Gordon (5) found that vaccine virus is most rapidly absorbed when injected subcutaneously or applied to the nasal mucous membrane. Gins (6) inserted cotton pledgets soaked with vaccine virus into the nasal cavities of rabbits and found that the animals were resistant to a subsequent intradermal inoculation, although no pathological alterations of the nasal mucous membrane resulted from the procedure. Le Gros Clark (7) investigated the route of absorption from the nasal mucous membrane and found that a solution of potassium ferrocyanide and iron ammonium citrate reached the surface of the brain within 1 hour by way of the perineural sheaths of the olfactory nerves. The perineural spaces were found to be continuous with the subarachnoid space above and to extend peripherally along the terminal fibers of the olfactory nerves to the sensory epithelium of the nasal mucous membrane.

It has been shown then: first, that vaccine virus is very rapidly absorbed when given subcutaneously or by way of the nasal mucosa; second, that it can produce immunity when absorbed from the nasal mucosa, and third, that free vaccine virus can be recovered from a mixture of the virus and its specific immune serum.

In view of these facts it has seemed desirable to ascertain the immunizing power of neutralized vaccine virus-immune serum mixtures given by the nasal and by the subcutaneous routes respectively.

Material Employed

A stock strain of Levaditi neurovaccine was employed which had been kept at a constant level of infectivity by glycerolation and fairly frequent animal passage. The activity of the strain was such that symptoms of vaccinal encephalitis appeared 3 days after intracerebral inoculation in rabbits. The animals were etherized as soon as convulsions were observed and the brain was removed with precautions for asepsis, cultured to exclude bacterial contamination, and ground with sand and physiological saline to make a 10 per cent emulsion. After brief centrifugation to throw down the sand, the supernatant fluid was removed by a pipette and diluted variously with saline. Normal rabbits were inoculated intradermally, on the one flank with 0.2 cc of each of the various dilutions and on the other with similar volumes of virus previously mixed with an equal amount of immune serum, obtained by bleeding rabbits 3 weeks after recovery from acute, pustular vaccinia. In most instances 0.2 cc. of immune serum sufficed to inactivate an equal amount of a 1:100 dilution of a virus which gave rise to lesions when inoculated alone in a dilution of 1:100,000. When this was the case, a 1:200 dilution of glycerolated vaccine virus brain mixed with an equal amount of immune serum could be considered neutralized material and this was used. Both the mixture and the glycerolated virus as such were inoculated intradermally into a normal rabbit as a

IMMUNIZATION AGAINST VACCINIA BY NON-INFECTIVE
MIXTURES OF VIRUS AND IMMUNE SERUM

By C. P. RHOADS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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The technical procedure employed in inoculating animals with vaccine virus as a prophylactic measure against the allied, more severe disease smallpox, has undergone few essential modifications since the time of Jenner. Methods of producing an equivalent degree of immunity without the lesions of pustular vaccinia have been attempted but have never proved completely successful. Recent investigations in the field of virus disease have pointed the way to a new experimental approach which appears to offer a means to the desired end.

The view, recently put forward by Andrewes (1), that a virus is not destroyed by contact with its specific antiserum but, on the contrary, forms a loose union from which it may be recovered, is the basis of the experiments reported in this communication. The method employed depends upon the use of vaccine virus and its specific immune serum mixed in such proportions that when the material is inoculated intradermally in a normal animal no lesion results though free virus is liberated in amounts sufficient to render the animal resistant to a subsequent injection of virus alone.

The use for immunization of a pathogenic agent neutralized or rendered innocuous by association with specific antistuff originates in the observations of von Behring (2) on diphtheria. He demonstrated that it was possible to immunize animals against diphtheria by the use of toxin-antitoxin mixtures. Arrhenius and Madsen (3) found that free toxin was liberated from the mixture but so slowly and in such small amounts that immune bodies were produced in the absence of disease symptoms. Andrewes (1) considered mixtures of virus and immune serum similar to combinations of toxin and antitoxin, since he was able to recover free virus from the mixture by dilution or by filtration through a Berkefeld candle. The same author (4) injected rabbits with vaccine virus and immune serum, coincidentally, into different veins, and found that 50 per cent of the animals so treated were immune to subsequent intradermal inoculation of virus alone. There

larly. After a 10 day interval, four of the animals were tested for immunity by the intradermal inoculation of 0.2 cc. of a 1:100, 1:1,000, and 1:10,000 of a salt solution dilution of fresh rabbit brain neurovaccine. A normal rabbit and one which had recovered from typical pustular vaccinia were used as controls. At the end of 24 hours, the treated rabbits showed a red, raised zone of erythema about 1.5 cm. in diameter at the site of injection of the 1:100 dilution. After 48 hours this reaction had faded, but the normal control exhibited characteristic elevated, firm, red lesions with blanched centers at all three inoculation sites. Necrosis was present in all these at the end of 72 hours, and from that time on the course of the lesions was typical of vaccinia pustule. Neither the treated animals nor the immune control showed any further reaction.

The same animals as well as two treated but uninoculated rabbits were tested for immunity 6 weeks after the final nasal instillation. The same dilutions of virus were inoculated and controls were like those in the series just described. Many of the treated animals showed a transient erythematous reaction to all dilutions, which reached its height in 48 hours and then rapidly faded. The normal control animal developed characteristic severe vaccinia lesions which progressed to necrosis.

Series B.—Four rabbits were treated in exactly the same way as those in Series A, except that after the third instillation one was etherized each day and an autopsy performed. Salt solution suspensions of brain, lung, and nasal mucosa were prepared and inoculated intradermally into normal rabbits. Those of brain and nasal mucosa uniformly gave rise to a vaccinia lesion, whereas lung usually failed to do so. The brain, nasal mucosa, lung, liver, spleen, kidney, and testicle were examined histologically and showed nothing abnormal.

RESULTS

The only visible reaction in the skin of the treated animals on subsequent test was a transient, raised, erythematous area, from 1 to 2 cm. in diameter, which developed only where the lower dilutions had been inoculated. This reaction ordinarily reached its maximum after 24 to 48 hours and faded rapidly. About 50 per cent of the treated rabbits showed this response, very similar to that resulting from the intradermal inoculation of animals known to be immune. Normal, control rabbits, on the other hand, developed erythematous areas at the sites of inoculation of all the dilutions and after a longer period of incubation. The lesions in the controls progressed rapidly through blanching to severe necrosis with subsequent extension, involving the entire flank of the animal, and often giving rise to generalized dermal vaccinia. A period of several weeks frequently intervened before healing was complete.

control for each immunizing treatment. Occasionally the total volume of the mixture was made up at the outset; at other times the virus and immune serum were mixed each time the neutralized material was used.

Intranasal Instillation

A series of treatments consisted of from three to five instillations at 1 or 2 day intervals. 1 cc. of neutralized material was dropped in each nostril at every instillation. Daily temperature readings were made and the animals observed closely to detect any nasal discharge or other pathological alteration. With certain groups of the treated animals were included rabbits which received nasal instillations of immune serum alone. Tests for immunity were carried out 10 days and 6 weeks after the completion of the treatments. For this purpose 1:100, 1:1,000, and 1:10,000 dilutions of fresh neurovaccine with physiological saline were prepared and inoculated into the treated animals, normal controls, and known immunes.

Subcutaneous Inoculation

In this group of experiments, the animals were treated by three subcutaneous inoculations of 2 cc. of neutralized mixture at intervals of 4 days. As in the series treated by nasal instillation, the material was glycerolated neurovaccine rabbit brain, neutralized with rabbit immune serum. Control animals received 0.4 cc. of the same material intradermally. No local reaction or rise in temperature was observed in either treated rabbits or the controls. Some were tested for immunity 10 days after the last inoculation and others after 6 weeks. The tests were carried out exactly as in the series treated nasally.

Certain animals were etherized at various intervals during and following the treatments. The organs, including nasal mucosa and brain, were fixed in Zenker's fluid, stained with eosin-methylene blue, and examined histologically. Nasal mucosa, brain, and lung were suspended and inoculated intradermally in normal rabbits. This was done to demonstrate that free virus had separated from the neutralized mixture *in vivo*.

ILLUSTRATIVE PROTOCOLS

Series A.—Ten rabbits were treated on 3 successive days by the nasal instillation of 1 cc. of a mixture of equal parts of 1:2,000 salt solution dilution of glycerolated neurovaccine brain virus and pooled rabbit immune serum. No local reaction or rise in temperature was observed. Some of the mixture was inoculated intradermally in a normal control rabbit at each treatment, and no reaction ever resulted. Injection of the unmixed virus gave rise to a characteristic lesion regu-

TABLE II
Result on Testing—Series Treated by Intranasal Instillation

Rabbit No...	Nos. 1-4 killed during treatment	5		6		7		8		9		10		Control		Immune control	
		Virus dilution		Virus dilution		Virus dilution		Virus dilution		Test delayed		Test delayed		Virus dilution		Virus dilution	
		100	1,000	10,000	100	1,000	10,000	100	1,000	10,000				100	1,000	10,000	
1	-	+	-	-	+	+	-	+	+	-				+	+	+	+
2	-	+	-	-	+	+	-	+	+	-				+	+	+	+
3	-	+	-	-	+	+	-	+	+	-				+	+	+	+
4	-	-	-	-	-	-	-	-	-	-				Necro-sis	Necro-sis	Necro-sis	+
5	-	-	-	-	-	-	-	-	-	-				Necro-sis	Necro-sis	Necro-sis	-

Interval of 6 weeks																	
day of second test		100		1,000		10,000		100		1,000		10,000		100		1,000	
		Virus dilution		Virus dilution		Virus dilution		Virus dilution		Virus dilution		Virus dilution		Virus dilution		Virus dilution	
		100	1,000	10,000	100	1,000	10,000	100	1,000	10,000	100	1,000	10,000	100	1,000	10,000	
1	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+
2	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+
3	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+
4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE I

Rabbit No.	1	2	3	4	5-10
1	Treated 1 cc. neutral mixture each nostril	Same	Same	Same	Same
2	Same	Same	Same	Same	Same
3	Same	Same	Same	Same	Same
4	Killed	Same	Same	Same	Same
5	Brain / Lung / Nose Histology neg. Reaction following i.d. inoculation normal animal	Same	Same	Same	Same
6	± - ±	Killed Brain / Lung / Nose Histology neg. Reaction following i.d. inoculation normal animal	Treatment discontinued	Treatment discontinued	-
7	++ - ++	± - ++	Killed Brain / Lung / Nose Histology neg.	-	-
8	Necrosis -	++ ++ Necrosis	Reaction following i.d. inoculation normal animal ± ± ±	Killed Brain / Lung / Nose Histology neg.	-
9	Fading Fading	Fading Fading	++ ++ Fading Fading	Reaction following i.d. inoculation normal animal + + + +	-
10	-	-	++ ++ Fading Fading	Reaction following i.d. inoculation normal animal + + + +	-

± transient erythema lasting not more than 48 hours, not associated with edema or induration.
+ erythema, edema, and induration lasting more than 48 hours.
++ erythema, edema, and induration lasting more than 48 hours.

+++ erythema, edema, and induration lasting more than 48 hours, not associated with edema or induration.

+++ erythema, often with blisters.

+++++ erythema, edema, and induration with local necrosis and definite spread.

... and induration of high degree may

ganglion, with marked necrosis involving a large area.

Tables I and II record a typical successful experiment involving nasal instillation of the neutralized mixture, and Table III the result of one with subcutaneous inoculation of similar material. The others were so similar as to render detailed description of them unnecessary. The animals treated by way of the nose showed a slight rise in temperature, rarely to above 103.5°. No febrile reaction was observed in the series of animals receiving subcutaneous treatments. The relatively small number of the latter in the series tabulated proved to be fully as effective as the greater number employed in earlier experiments.

The most suitable combinations of virus and immune serum were ascertained only after several trials. It was found that if insufficient serum was employed, a typical delayed vaccinal lesion resulted when the mixture was inoculated intradermally in a control animal. If an excess of immune serum was present, the immunity resulting from the treatments was slight, as compared with that observed in more favorable experiments.

In certain instances an amount of neutralized mixture sufficient for the entire series of treatments was prepared and stored at +4°C. When this was done, there was a well-marked tendency for free virus to dissociate from the virus-antiserum combination and produce vaccinia in the control animals. If this dissociation occurred, the treatments were immediately discontinued and the series was discarded. Animals treated with insufficiently neutralized material developed a sharp febrile reaction and a variable amount of nasal discharge, and their organs often showed typical lesions of generalized vaccinia when examined histologically. The changes were most marked in the lungs and were similar to those described by Stewart and Duran-Reynals (8) in a study of the effects of generalization of vaccine virus from enhanced skin lesions. It seems probable that the instillation of active virus into the nose, as practiced by Gins (6), depended on the presence of vaccinia lesions for the production of immunity.

Histological examination of the nasal mucosa, brain, lung, spleen, liver, and testicle of rabbits treated with properly neutralized material showed no vaccinal lesions. Virus could often be recovered from the nasal mucosa and from the brain and occasionally from the lung.

TABLE III
Result on Testing—Series Treated by Subcutaneous Inoculation

Rabbit No...	Nos. 1-4 killed during treatment	5		6		7		8		9		10		Control		Immune control	
		Virus dilution		Virus dilution		Virus dilution		Virus dilution		Test delayed		Test delayed		Virus dilution		Virus dilution	
		100	1,000	100	1,000	100	1,000	100	1,000					100	1,000	100	1,000
day of first test																	
1	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
2	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
3	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
4	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
5	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
Interval of 6 weeks																	
day of second test																	
1	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
2	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
3	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
4	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+
5	-	100	1,000	100	1,000	100	1,000	100	1,000					+	+	+	+

This is in accord with the observations of Demme (9) which indicate that active vaccine virus may be present in tissues without giving rise to lesions.

CONCLUSIONS

1. Vaccine virus and specific immune serum mixed in such proportions as to produce no lesion when inoculated intradermally induce immunity when instilled into the nasal cavities of rabbits.
2. The mixture is also effective when inoculated subcutaneously.
3. Immunity is brought about with a minimum of systemic reaction and no local one.
4. When the mixtures are incompletely neutralized generalized vaccinia results.

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since the arrangement of the vessels proved relatively unfavorable to the aim in view.

Despite many efforts we have been unable to find conditions under which a good circulation in the exposed tissue can be long maintained, or even assured to begin with. The practical absence of staining of the thigh muscle of unanesthetized frogs which behaved normally after the dye injection, leaping about when touched, proved that even in them the local circulation was insufficient to distribute the dyes in quantity. This can be attributed, in part, to posture,—for the muscles we examined bore much of the weight when the frog was quiet,—but mostly to the poor normal circulation. Krogh states that in resting frog muscle the capillary circulation is very variable and as a rule feeble, especially in the limbs (2). So often was the blood flow found to be highly irregular or nearly at a standstill on exposure of the muscle that we were forced to sacrifice frog after frog in order to obtain individuals in which, for reasons unknown, flow was good. Once it stopped in the exposed muscle, it practically never was resumed. Acting on the possibility that its cessation after pithing or anesthetization might be merely transient, we kept some animals for an hour or more thereafter before exposing the muscle. In them the circulation was more frequently at a standstill than in others opened at once.

The best preparations were obtained by injecting curare into a dorsal lymph sac (0.1 cc. of 1 per cent curare for a 30 gm. frog), pithing the brain immediately that the animal had become flaccid, and laying bare the muscle. The animal was placed on its back upon a moist cloth at the bottom of a shallow observation dish. Some frogs were pithed in both brain and cord; these in general had a poor local circulation which soon became worse irrespective of whether the tissue was exposed. Others were only curarized or etherized. The dye injection caused the muscle circulation to stop in some animals whereas in others receiving the same material it was unaffected. As a rule the skin circulation was brisk, no matter how poor that in the muscle.

The Vascular Arrangement in Frog Muscle

The arrangement of the muscle vessels is easily discerned in the sartorius (Fig. 1). It can be made out nearly as well during life as after the injection of colored masses.

In *Rana pipiens* the large vessels enter the muscle from beneath and ramify much more irregularly than in mammals. The larger venous tributaries connect with others of the same magnitude by collaterals, and the final arterioles and venules lie transverse to the muscle fibres, those in the same plane alternating at a distance of 1 to 2 mm., the gap being bridged by numerous capillaries in parallel. The venous twigs have an espaliered arrangement which appears slightly crowded as compared with the ramification from the corresponding arterioles.

THE GRADIENT OF VASCULAR PERMEABILITY

II. THE CONDITIONS IN FROG AND CHICKEN MUSCLE, AND IN THE MAMMALIAN DIAPHRAGM

By FREDERICK SMITH, M.B., B.Ch., AND PEYTON ROUS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 8 TO 10

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Experiments already reported (1) have shown that the permeability of the capillaries in the skeletal muscles of mammals increases progressively along their course and is greatest where they pass into the least venules. In the present paper we shall describe the state of affairs in frog and chicken muscle and in the mammalian diaphragm.

The Technique with Frogs

The same general methods were employed as previously. The spread of dyes from the blood was watched directly in muscle, with a binocular microscope, by the cooled light from an arc lamp. Ordinarily the tissue was exposed by cutting through the skin of the anesthetized animal from just above the knee to half way up the thigh on its anterior, outer aspect, and continuing the incision at right angles, to the inner side of the limb. On reflecting the triangular flap of skin thus loosened, portions of the sartorius, gracilis major, and crureus were laid bare, usually without the least bleeding. The muscles on the foreleg were occasionally studied; but those of the back and abdomen are so overlain with fascia and pigment cells as to be unsatisfactory. Owing to the large lymph spaces beneath the skin the mica windows used to protect mammalian muscle from drying would not stay in place. Occasionally the leg was submerged in frog Ringer's solution during the examination, but under such conditions less consistent results were obtained than when a thin sheet of cellophane (du Pont No. 350, 400, or 600) softened in Ringer's solution had been laid directly upon the exposed area. The sheet was kept moist with the solution, which also minimized reflection from its curved surface.

As control to the findings in anesthetized animals, numerous others were injected with dye and decapitated after various intervals. Summer frogs of two species were employed, *Rana pipiens* and *Rana clamitans*, the latter to but small extent

Procedure with the Dyes

A more various set of dyes were employed than in the work with mammals. All had been freed of the extraneous matter which in not a few instances constituted a large proportion of their bulk. They were in watery solution isotonic with frog blood,—on the assumption that this itself has the tonicity of 0.7 per cent NaCl. The solutions had in some cases been brought to approximately the pH of blood but in others this precaution was not taken since the dye had no buffer value and the difference from the blood pH disclosed by the potentiometer was slight. The injections were made into the dorsalis pedis vein of the leg that was not to be studied. When the needle had been thrust for a little distance through the fascia before entering the vein at the junction of the venae tarseae lateralis and medialis, no bleeding followed. Magnifying spectacles or a watchmaker's glass were required if the vessel was to be entered regularly. Trial showed that unanesthetized animals yielded the most informative muscle staining if they were killed as soon as the rugose skin on the inner thigh surface next the crotch had stained deeply.

The Passage of Dyes from the Blood

Far less dye passed into the muscle than into the skin and some other organs; and the more poorly diffusible the material the greater this difference proved to be. Only by giving large amounts of pontamine sky blue could one obtain an informative muscle staining before escape of the dye elsewhere had reduced the concentration in the blood below the effective level; while in the case of Congo red this end could not be achieved with the largest tolerable dose.

As one might expect from the abundant lymph formation in the frog (3) the passage of dyes from the blood is far more rapid than in the rabbit or guinea pig (1). Patent blue V and phenol red escaped at once all along the capillaries as the stained blood advanced through them, and local differences in rate of escape were not discernible with their aid. Such differences were readily brought out with less diffusible stains (Fig. 2).

SPECIMEN PROTOCOLS

Eosin (Yellow).—This dye belongs to a group not utilized in our previous work. The Grubler material (Dr. Karl Hollborn) was purified by extraction with absolute alcohol in the cold and evaporation on the steam bath in a strong current of air at 55°–60°. A 4.3 per cent solution in water was found by freezing point determinations to be isotonic with frog blood. The progress of the staining was most readily followed in light filtered through water tinted with methylene blue.

The walls of all of the vessels are invisible in the living animal and, when these are empty, the tissue appears avascular. The capillaries are further apart and generally wider than in mammals; and their individual calibre as shown by their content varies greatly, a continuous column of blood several red cells thick flowing through some, while through others individual cells pass intermittently. All along their length the diameter is the same, but their number undergoes an increase in the further half of the region between arteriole to venule, owing to forkings that take place about midway (Fig. 1). In this respect there exists a striking similarity to mammalian muscle; but anastomoses between adjoining capillaries are somewhat more frequent, being met especially along their further portion, while furthermore some secondary forking may take place as the venules are neared, and these vessels are wider than the corresponding arterial twigs. Because of the resulting increase in the vascular bed, preparations injected with India ink and cleared appear slightly darker in the neighborhood of the transverse veins. It is frequently possible to find regions in which the capillarization is almost equally abundant everywhere between arteriole and venule, and such have been selected for our observations.

When the circulation is good, the whole field under the microscope appears at first view to be sliding rapidly in one direction or the other. Often, however, the blood barely creeps along. We have utilized many instances of both sorts. Stasis is readily discerned, and so too is unnatural congestion. In the crureus and adductor magnus, the vascularization, while more irregular than in the sartorius, has the same general arrangement; and in them circulation is especially well maintained after exposure. Their arterioles and venules lie relatively near together so that it is possible to watch several sets of capillary vessels at once under relatively high power.

Stained specimens show the capillaries to be walled by a single layer of flat cells with very flat nuclei. There are no obvious structural changes along their course. Direct arteriovenous anastomoses are occasionally seen; in fact the very large capillaries can almost be considered as such.

In *Rana clamitans* the transverse arterioles are far less regularly distributed than in *R. pipiens* and they break up into numerous twigs, each furnishing a few capillaries to the muscle. The venous trees are also relatively complicated. While the ultimate arterial or venous twigs from one transverse vessel usually lie near together, yet there is sufficient intrusion of those of the opposite sort from adjoining planes to render observations difficult, a difficulty added to by the shortness of the capillaries. The division into the latter takes place somewhat below the surface of the sartorius as a close arborization, and one sees only the final forks of the arteriole which go off at rather an obtuse angle. As in *pipiens* relatively few capillaries traverse the arteriolar tree from side to side, a fact which indicates, like our previous observations (1), that an effective exchange with the tissue takes place through the walls of the arterioles themselves. The number of capillaries increases considerably as the venules are approached.

seen to escape everywhere from the capillaries, blurring them. Escape from the smallest venules took place at the same time. The coloration rapidly became diffuse. Within a minute, however, a barring with deeper color of the same general extent, situation, and graded character as that with eosin was superimposed upon the general color. After 90 seconds in all a special zone of color had formed just outside the transverse collecting venules, manifestly as result of the direct escape of dye through their walls. After little more than 2 minutes the staining in the barred region had completely obscured the capillaries there. By now the circulating quantity of brom phenol blue had so greatly diminished that the individual corpuscles could again be discerned. Nevertheless the bluish purple, diffuse staining underwent some further intensification, and in the next few minutes the bars were gradually lost in it. There was no zone of special staining along the large veins into which the transverse collecting venules gave.

The findings with brom phenol blue were like those with eosin except for a special staining along the venules, which may well have taken place with eosin but not have been discernible.

Trypan violet has been used but little.

In a curarized *Rana pipiens* of 46 gm. given 0.4 cc. of 2.9 per cent trypan violet in 57 seconds, the dye appeared in the muscle vessels within half a minute, and at the conclusion of the injection had begun to escape all along the further half of the capillaries, in increasing amount as the venules were neared. After 2½ minutes altogether a narrow zone of especially deep color could be seen just outside these latter. A violet barring in the distal capillary region was now very pronounced in the gross. Under the microscope the tissue appeared striated with color because of the stain outside individual capillaries, which extended further back along some than along others, with result that the margins of the bars had a step-like irregularity. The circulation continued excellent.

Trypan Red (Vital Red HR).—This proved highly satisfactory save in that it tended to do away with the paralysis of curare. It was well tolerated, readily followed in its extravascular spread with the aid of the methylene blue filter, and it did not obscure the blood corpuscles to such extent but what all changes in flow could be perceived.

Practically at once after the injection—0.3 cc. of 2.8 per cent dye for 50 gm. of frog, in 30 to 45 seconds—the further portion of each patent capillary became surrounded by a fuzz of color that, rapidly deepening, encased and blurred it. Extravascular dye was first visible in the region of the venocapillary junction, and then further back along the capillary. The distribution of stain, unlike a dye ecchymosis, was symmetrical, taking the form of a colored sheath which widened toward the venule. Along the proximal half of the capillary no staining occurred nor was there any about the arterioles. By the end of 2½ minutes the extravascular dye had spread laterally and become confluent. The muscle now was red everywhere

In animals with a brisk blood flow, as indicated by the almost instantaneous passage of the stained blood from arterioles to venules, the escape of eosin was so rapid everywhere along the little vessels that often the tissue next them appeared to color everywhere at the same rate. Resort was had for this reason to frogs in which a sluggish though continuous circulation was found, as when both brain and cord had been pithed. In these a series of phenomena were noted like those in rabbits injected with the very highly diffusible patent blue V after a large bleeding to lower the blood pressure and slow the local circulation (1). No doubt exists that in the frog the sluggish circulation was the result of a low pressure, for the signs of venous obstruction, capillary contraction, or developing stasis were wholly lacking. Some of the capillaries were empty, that is to say invisible, but many had the normal breadth, and flow in them was continuous.

0.2 cc. of eosin solution was injected for 40 gm. of frog in the course of from 10 seconds to a minute. After about half a minute deeply stained blood appeared in the terminal arterioles and dye could be seen to pass out at once from these vessels into the surrounding tissue and then from the capillaries as the stained blood flowed along them. Most of the eosin left the blood during the short journey, appearing almost to pour from the slender channels, and an interval elapsed,—of more than 20 seconds in one animal,—before enough of the stain entering the capillary reached its further end to color the plasma there definitely. At the end of about a minute and a half the muscle had become evenly pink everywhere. But now a transient, superimposed barring with deeper color developed, which was lost within another 2 or 3 minutes as the diffuse coloration intensified. The bars had venules at their center and they extended with diminishing intensity half way back to the arterioles.

There could be no doubt that eosin got out from arterioles as well as from capillaries; these latter proving highly permeable everywhere along their course. The tissue first reached by the stained blood was first served with dye by it, yet nevertheless an even staining soon developed, and secondarily there was superimposed upon this an especially intense coloration of the region traversed by the further portion of the capillaries and supplied with blood which was relatively poor in dye, owing to the continued escape of it along the proximal portion of the little vessels.

Brom Phenol Blue.—The spread of this substance in the tissues, as of other blue or purple dyes, could be followed in greater detail than that of red ones. No color filter was needed.

Usually 0.25 cc. of 2.7 per cent solution for 35 gm. of frog was injected in 20 to 30 seconds. When the local circulation was brisk the dye reached the muscle venules an instant after it appeared in the arterioles, and practically at once was

Chicago Blue 6B.—The escape from the vessels of this poorly diffusible dye was so gradual that under the microscope one saw only a very gradually developing extravascular mist in the region of the further portion of the capillaries.

Frogs of 35 gm. were given 0.1–0.2 cc. of 5.8 per cent solution in the course of a minute. Soon gradually deepening bars of blue were noted in the gross. Each had a transverse venule as its axis. All were extremely narrow, and there was but little opportunity for them to widen secondarily, since by the time they had developed the blood was largely depleted of the dye by its escape elsewhere. In unanesthetized frogs pithed for examination 5–8 minutes after dye injection, the barring was found to be superimposed upon a pale, diffuse staining. There was no localized zone of color immediately next the venules to suggest passage through their walls.

As already mentioned, dark dyes tint the plasma so deeply as to obscure the corpuscles. The blood flow can no longer be perceived except when cells containing black pigment are in circulation, as occasionally happens. The hopping course of these through the capillaries then attests to a general movement. Ordinarily, though, all the vessels appear as if rigid with stained injection mass, and only after considerable decolorization of the plasma has occurred can one see that circulation is going on. For this reason we have felt obliged to carry out many experiments with *Chicago blue 6B*. As might have been expected from its poor diffusibility (4) it escaped into the tissues to a very limited extent, and almost entirely from the far ends of the capillaries.

Pontamine sky blue 6B (du Pont de Nemours) is listed in Rowe's Colour Index (7), as identical with *Chicago blue 6B* (General Dyestuff Corporation). The crude dye stained mice far more slowly than *Chicago blue 6B* and the animals took on a blue-green color instead of a clear blue. This was true as well of the purified dye.

The crude material was purified like *Chicago blue*, by dialysis through Reeve Angell paper ("diphtheria parchment") against water at room temperature; but its salt content caused such an increase in bulk that reconcentration had to be carried out after about 20 hours, before purification was finished. Since no preservative had been added, the concentrate was heated at this time to just below the boiling point for 15 minutes to sterilize it. After a second dialysis of 24 hours, first against running water and then distilled, the material was evaporated on the steam bath.* Tests now showed it to be free from extraneous matter.

* Needless to say, the process may have polymerized the dye. Such a change, if it occurred, was advantageous as broadening the conditions of experimentation.

throughout the further capillary region, whereas nearer the arteriole there was still no staining. Thus there developed transverse bars (Fig. 2) which persisted for a considerable time. After a few minutes a narrow zone of intenser color developed next the small venules as if by direct escape of dye from them; but nothing of the sort was to be seen outside of the larger veins. Not infrequently situations could be found in which capillaries entered a venule on but one side; and only the tissue on this side was colored. Frequently when the circulation was vigorous, the red bars were superimposed upon a pink, generalized staining. They then appeared narrower, their limits being lost in the coloration of the rest of the tissue. In the gracilis major, where branching venules and arterioles are almost superimposed in adjoining planes, the color soon spread from one plane to another, confusing the picture.

The variations from the results with eosin and brom phenol blue were such as might have been expected, on the basis of previous work, from the slighter diffusibility of trypan red (1). Its escape from the further portion of individual capillaries as a fuzzy sheath or fringe, increasing in intensity as the venule was approached, duplicates observations frequently made with eosin and trypan blue, but not so readily to be interpreted in their case since they obscured the blood flow to such extent that intercurrent venous obstruction could not be ruled out as a possible cause for the phenomenon.

Trypan Blue (Grübler-Hollborn).—Trypan blue has a very different formula from trypan red, but nearly the same diffusibility (1). According to Schulemann (4), it kills when in Ringer's solution but is well tolerated in distilled water, as our preparation was. Large doses cause hemolysis (5). Dye ecchymoses were more often noted with it than with any other of our test materials, while furthermore the circulation in exposed muscle stopped with especial frequency after its injection. In the amount used by us it elicited no symptoms in unanesthetized frogs.

0.25 cc. of a 4 per cent solution was injected for 35 gm. of frog, in from 1/2 to 3/4 minute, rendering the blood so dark that the circulation seemed at a standstill. Well pronounced blue bars had developed ordinarily within 1½ to 3 minutes in the region traversed by the further third of the capillaries. The dye was seen to escape directly from the vessels in this region before it did from the venules into which they gave. In frogs with active circulation the barring was frequently superimposed upon some pale diffuse staining which had a pinkish cast referable to the presence in the dyestuff of a ruddy component more diffusible than is the blue one (6).

The findings closely resembled those with trypan red and trypan violet.

of dye that would disclose the local differences. Whether the muscle fibres themselves took the stain was an immaterial matter. It was enough for our purposes to be able to perceive where and when the coloring matter got out into the tissues.

The findings were consistent despite the highly various circumstances of the work, and were nearly identical with those in mammals (1). They appear to provide evidence that there exists as in these latter a mounting gradient of permeability along the course of the capillaries, which reaches its height at the junction with the venules and falls away rather slowly along the latter. Yet this evidence must be scrutinized further before such a conclusion is justified.

That the coloration resulted from passage of dye into the tissues is certain since the mere filling of the vessels with stained blood did not produce it. Where capillaries carrying a rather poorly diffusible dye entered but one side of a transverse venule the staining of the tissue was limited to this side, a fact which indicates that the escape of such dyes occurred from the capillaries only.

As already mentioned, the capillaries usually undergo some numerical increase as the venule is approached; and the question arises of whether the intenser staining hereabouts may not have been due merely to a larger vascular surface through which exchange might take place, irrespective of any local difference in permeability. And there is still another possibility to account for the localized coloration in the case of easily diffusible dyes. These escape in abundance through the walls of the smallest venules, a fact attested by the development of a narrow zone of deep color next them. Often when the circulation is sluggish almost no blood flows through the individual capillary, yet enough in the aggregate for a slow current in the venule. Under such circumstances a considerable staining may take place from it while that from the capillaries will be negligible. We have frequently observed this occurrence; and though the dye escapes only from the venule it spreads secondarily with surprising ease. Indeed even in animals in which the circulation has stopped immediately after the dye-stained blood has entered the muscle, and the capillaries have contracted later, squeezing the blood into the venules, the tissue next the latter colors and the color soon extends backward toward the arterioles.

We have been able to appraise the importance of the increase in wall surface as the venule is neared by the selection of regions for observation in which this increase was practically absent. Here the same graded extravascular coloration took place along the further course of the capillaries as elsewhere in the muscle. Such coloration could not have arisen by a secondary spread backwards of poorly diffusible dyes, since to them the venules proved impermeable; and in the case of dyes more diffusible it developed by the lateral extension and coalescence of a colored mist which formed around each capillary through which the stained blood passed.

Pontamine sky blue thus purified stands at the extreme limit of those dyes with which a visible vital staining of frog muscle can be accomplished. A 16.5 per cent solution is required for isotonicity with frog blood,—as compared with 5.8 per cent for our preparation of Chicago blue. Not a little dye deposits out of this solution during the routine filtration just prior to injection, and hence we have ordinarily used the material in half strength, an 8.25 per cent solution in 0.35 per cent saline. 0.2 cc. of this was well tolerated by a 35 gm. frog when injected in about a minute. The muscle vessels stood forth immediately as in a line drawing with deep blue ink, and after the lapse of several minutes the dye appeared in the tissue about the further end of the capillaries, and intensifying formed sharply defined, very narrow blue bars. Meanwhile the blood lost color. No diffuse coloration occurred, and no zone of special staining appeared next the transverse venules lying in the axis of the bars.

Pontamine blue gets out into muscle only from the further end of the capillaries.

Other Dyes.—Congo red and methemoglobin (of the horse) failed to escape in perceptible quantity. We were led to try the latter because of the permeability of frog capillaries for blood proteins (3).

Findings confirmatory of the foregoing were obtained in the few experiments upon *Rana clamitans*.

Analysis of the Results in Frogs

A gradient of dye distribution along the course of the small vessels proved readily demonstrable in frog muscle. The findings depended to no inconsiderable extent, however, on the amount of dye injected. When this was small and the substance readily diffusible (eosin, brom phenol blue) an even staining took place along the capillary way; while with more of the same material a barring of intenser color, in the region traversed by the further portion of the capillaries, was superimposed upon the diffuse coloration. When small quantities of poorly diffusible dyes (Chicago blue, pontamine sky blue) were injected none escaped into the muscle, though a great deal passed into the skin and some other organs; but with more a narrow barring of the muscle developed, one localized to the furthest capillary region; and when much had been thrown into the blood some pale, general staining of the muscle took place besides the barring, save in the case of the almost indiffusible pontamine sky blue. We have as a rule employed the least amount

Character of the Staining in Chicken Muscle

Very different were the findings in chicken muscle.

Well nourished young Plymouth Rock pullets of 1100–1300 gm. were used for the work. They were etherized; a circular piece of skin was snipped out of a featherless area with careful avoidance of large vessels; and an oval, mica slip was inserted which fitted the opening snugly and lay against the muscle sheath. The skin is so loose in fowls that such a window can be pushed about with it over a large area, exposing many fields successively. Thus, for example, when it is placed at the lower edge of the thorax in the axillary line, either the pectoralis major or the external oblique can be observed at will. If the window is introduced further down over the oblique the entrance of air into the subcutaneous tissue is difficult to avoid. Immediately after the insertion the dye was injected, usually into a wing vein; and sometimes new windows were put in whilst the staining was going on. When a pectoral was to be watched the wing was let rest on a board, to avoid stretching the muscle. Staining was checked by decapitation, and layers of the muscle were rapidly separated out and inspected between glass plates under the binocular microscope. The thin sheet of the external oblique, removed *in toto*, proved especially suited to study, the relationship of arterioles, capillaries, and venules being almost diagrammatically visible (Fig. 4).

Chicago blue 6B, trypan red, trypan blue, brom phenol blue, and patent blue V were injected, in the proportions for body weight which had given clear cut results with mammals. The fowls were killed at times which seemed most favorable to the disclosure of local differences in vascular permeability.

The muscles always stained much less than those of rabbits. With trypan blue, for instance, which colors rabbit muscle brilliantly and promptly, almost no staining occurred in 20 minutes, so little dye passing from the blood that the pectorals appeared only pale green and the hemoglobin-containing muscles green-brown. By this time some of the other tissues (skin, gastro-intestinal tract) were deep blue, and the quantity of dye in circulation had so far diminished that but slight further coloration of the muscles could be hoped for. With trypan red the staining was also relatively slow and slight. Chicago blue 6B, a far less diffusible material (1), caused only the slightest muscle staining in half an hour; but not enough escaped elsewhere to reduce the concentration in the blood below the effective level and in the course of 2 hours the muscle became gradually and diffusely dark blue. Chicago blue yields in etherized rabbits a deep blue barring after 15 to 20 minutes. Brom phenol blue, which is fairly diffusible, gave a definite coloration of chicken muscle after 5 minutes; but the

Final evidence of the existence of a gradient of permeability along the capillaries was obtained by observing directly the escape of dye through their walls. The fact that their individual calibre, be it large or small, is nearly the same all along should be emphasized in this connection. Some dyes (eosin, trypan red, trypan blue) could be seen to spread rapidly outwards from the blood, surrounding the wall through which they passed with a fuzzy sheath of deep color and soon hiding it completely. This sheath appeared first and was always most pronounced at the further end of the slender vessel, thence tapering and fading in the direction of the arteriole. Needless to say, the possible influence of intercurrent passive congestion and of abnormal capillary dilatation to account for the phenomenon had to be kept in mind. Sometimes its absence from muscle regions newly laid bare attested to abnormal vascular conditions in the tissue already exposed; and comparison showed that the capillaries of the latter were dilated. Abnormal capillary dilatation is known to increase permeability (8).^{*} The fact deserves remark that the greatest escape was localized to the distal portion of the dilated vessels, though pronounced elsewhere. When all pathological instances had been excluded there remained a sufficiency in which a graded escape of dyes along the capillaries was directly noted. It took place deep in the transparent muscle as well as superficially. Trypan red yielded especially convincing results because any abnormalities of the blood flow could be readily detected despite the staining of the plasma.

The conclusion seems justified that the walls of the capillaries of frog muscle become increasingly permeable along their further portion. Whether a graded permeability extends back all the way to the arteriole our experiments have not disclosed, owing to the circumstance that the dyes which would be most affected by differences in the proximal capillary region get out so quickly everywhere that differences in the time at which they reach this point and that overwhelmingly condition the color phenomena. The fact that when such dyes are given in small amount their distribution along the capillaries is an even one, despite the lessening amount in the blood as it advances through the little vessels, indicates that permeability increases all along the latter to an extent that offsets the diminution just mentioned. This conception has already been brought forward in a paper on vascular permeability in mammalian muscle (1).

^{*} Ecchymoses of dye are sometimes encountered in muscles that have been clumsily exposed. They are easily recognized, the dye escaping irregularly here and there, often from one side only of a vessel and in such great quantity as is never seen on ordinary occasion.

3:51—No muscle staining; vessels brilliantly demarcated, without abnormal dilatation or contraction. 3:52—Skin has turned blue. The muscle capillaries are easily to be seen. 3:53—There is a medium blue, perivascular staining about the large vessels emerging through the sheath of the pectoralis. 3:53½—Eyelids deeply blue. 3:54½—Muscle staining blue evenly. 3:59—The skin is rapidly becoming a darker blue. Interior of mouth brilliant purple blue. 4:00—The larger muscle vessels are now emphasized by a blue staining of the perivascular connective tissues. 4:04—Muscle itself undoubtedly darker blue. 4:13—Skin deep blue. The muscle is darker and the thick fascial septae and the perivascular connective tissue stand out in darker blue. 4:18½—The muscle appears evenly stained, between *deep Dutch blue* and *slate blue*.

4:20—Killed. The external oblique, spread between plates and examined under the binocular microscope, shows everywhere a brilliant staining of the connective tissue about the larger vessels, and staining of their walls as well. Within the muscle itself the hue is even. So too in the pectoralis major. The alimentary tract is a deep, uniform purplish blue and contains a small amount of intensely stained mucus. The lymph is moderately blue, the liver reddish purple. The bladder bile is nearly if not quite as deeply colored as the solution used for injection.

Patent Blue V.—A Plymouth Rock chicken of 1100 gm. was etherized at 2:10; between 2:33–37 windows inserted; and at 2:47 the injection was begun of 3.3 cc. of patent blue V solution isotonic with the blood. The injection took 9 seconds. Meantime the external oblique was watched. It showed the vascular arrangement diagrammatically. 5 seconds after beginning the injection the blood in an arteriole under observation had become brilliantly blue, whereas it was still bright red in the accompanying venules. Immediately thereafter the muscle began to color diffusely everywhere, the dye leaving the plasma so completely that the venous blood continued to be ruddy. The hue of the muscle darkened fast, and 11 seconds after the end of the injection the animal was decapitated. By this time the venous blood had become perceptibly blue; but it was much less deeply colored than that in the arteries.

Both the pectoralis and the external oblique were now an intense and even blue-green. The latter muscle was rapidly removed and inspected between slides. The pectoralis major showed suggestions of a colored barring in the gross, but this proved referable merely to intense staining of the connective tissue surrounding the larger vessels. The intestines were deeply stained, and so too with the skin, the latter being more intensely colored than with Chicago blue 6B after several hours. The bladder bile was deep blue, and the liver dark with stain.

Other instances need not be given. Always the muscle tissue appeared diffusely colored in the gross. There was a complete absence of the barring which in mammals and frogs is expressive of a mounting gradient of permeability along the muscle capillaries.

color was pale as compared with that in the rabbit. Only with the very highly diffusible patent blue V was there intense, prompt staining.

These findings in fowls cannot be explained by circulatory difficulties or by lack of circulating dye. For the rapid passage of the latter into and through the muscle vessels was plainly visible; and intense staining of the skin and gastro-intestinal tract soon took place, with elimination into the bile. Furthermore there occurred a staining of the perivascular connective tissue within the muscle, accentuating the vessels.

Some typical findings will be given.

SPECIMEN PROTOCOLS

Chicago Blue 6B.—An 1100 gm. chicken was etherized at 10:40 a.m., and at 11:05 a mica window was inserted over the pectoralis major. The muscle appeared almost colorless under the binocular and beautifully transparent, with the circulation going on as if through channels in jelly. 11:15—There has been no local vascular dilatation since the muscle was exposed. 11:16½—Injection begun into a wing vein of 3.3 cc. 8 per cent Chicago blue 6B, in 3 minutes and 47 seconds.

20 sec.—The dye has reached the muscle and practically at once all its vessels stand out sharply in dark blue. 4 min.—The exposed tissue appears very pale blue in the gross. 7 min. 10 sec.—Muscle now evenly tinted, between *artemesia green* and *lily green* (9). 12 min.—The colored muscle is still transparent; the capillaries can be only dimly made out.

11:34—Skin green-blue, darkening gradually; 11:45—Muscle color approaching *lily green*; 11:48—Voided much dark blue, semi-fluid material. 11:51—Pectoralis darker and bluer than *lily green*. There is blue staining of the tissue surrounding a large artery and vein which emerge from the pectoralis to run on its superficial surface, as further some coloring of the intramuscular perivascular fascia. 12:08—Muscle evenly colored, darker than *dark Medici blue*. 12:33—Muscle between *dark Medici blue* and *dark green-blue slate*. The vessels are still brilliantly outlined. 12:45—The intramuscular connective tissue septae have gradually taken on a brilliant blue color. 1:15—Muscle everywhere of the same hue, between *Saccardo slate* and *dark green-blue slate*. The blood is less stained than before.

1:22—Decapitated. At autopsy the exposed pectoral and its control had the same even coloration, the lungs were unstained, the liver deep purply blue, the bile deep blue, and the intestines themselves deep blue and full of deep blue fluid. The gizzard was purply blue.

Brom Phenol Blue.—A 1300 gm. pullet was etherized at 3:30; between 3:45–47 two windows were inserted over the pectoralis major; and at 3:50 the injection was begun of 4 cc. of 4 per cent brom phenol blue solution. The dye was given in 7 seconds. It reached the muscle arteries 2 seconds before the conclusion of the injection and appeared in the veins immediately after.

The Vascularization of Pigeon Muscle

The presence of two identical, very abundant and close-knit vascularizations, providing flow in opposite directions amidst the fibres of chicken muscle, attests to the necessities of the working organ, perhaps in the past when the fowl was a flying creature. However this may be, it is interesting to inquire into the vascularization of muscles responsible for long sustained, rapid flight. We have studied therefore the pectorals of the pigeon. The arrangement in them bespeaks functional urgencies very clearly.

Pigeon muscles are too dark for the distribution of dyes in them to be readily seen. Our preparations were injected with colored mass and cleared. As in the chicken the final transverse venules and arterioles appear to pass to the tissue side by side in parallel; but by careful teasing and abrasion of fixed material, it is possible to isolate individual very shallow layers of muscle with vessels intact, and then one sees that venules and arterioles really alternate (Fig. 7). The distance to be bridged by the capillaries is exceedingly short, not over 0.2 mm. at most, but it is increased in the case of about half of these vessels by an abrupt change in their course so that they converge upon the venule in a long, narrow fan, enlarging slightly while so doing (Fig. 7). The capillary fans alternate with arteriolar trees which have the same simple structure as those in the muscle of other animals.

The Gradient in the Mammalian Diaphragm

The continuous functioning of the mammalian diaphragm suggested an examination of this muscle.

As might be expected from the demands upon the diaphragm, its blood supply is especially rich, and it is always succulent with lymph. Early investigators noted that it stains far sooner and deeper with vital dyes than most skeletal muscles. Open capillaries are more numerous than in the latter, under ordinary conditions (2), and the circulation is excellently maintained and vital staining intense even after bleedings which result in so great a contraction of the vessels in other muscles that these fail to color at all (10).

The vascularization of the diaphragm has been described by Spaltcholz (11), but into its minute features he did not go. It shows the same alternation of transverse arterioles and venules as other mammalian muscles but the vessels are nearer together, being little more than 1/2 mm. apart in the rabbit as compared with more than 1 mm. in the gracilis. A significant feature is a sudden, large increase in vascular area near the venules. Many of the capillaries fork about half way from the arteriole, as is usual in skeletal muscles, and thus their wall area is expanded; but in addition some of them enlarge near their end and turn and course alongside

The Vascular Arrangement in Chicken Muscle

A study of the arrangement of the vessels from which the dyes were distributed to chicken muscle showed why the findings were at variance with those in mammals and the frog. We used both fresh material and tissue fixed after injection with colored masses (Figs. 4 and 5).

The vessels of chicken muscle are much more abundant and minute than those of even the mouse. The slenderness of the final arterioles and venules at once attracts attention. In mammals and the frog these lie separate, in regular alternation transverse to the muscle fibres, the gaps between them being bridged by parallel capillaries. The latter are from 0.75 to 1.5 mm. long in the frog and rabbit, and their graded permeability results in a barred staining, the center of each bar being separated from the next by the distance between two venules, by 1.5 mm. to 3 mm. that is to say. In the chicken on the other hand the final arterioles and venules, while lying transverse to the fibres, do not alternate at considerable distances but run in parallel next each other, often two venules with an arteriole (Figs. 4 and 5). Each of the latter breaks up into a very small group of capillaries, and these, instead of emptying into the adjacent venule, course to another at a distance of $1/3$ mm. or less. Immediately next this venule, however, is an arteriole giving off capillaries which run to the venule situated next the arteriole first mentioned. Thus it comes about that throughout the muscle small adjoining groups of parallel capillaries carry the dye-containing blood in opposite directions. These details can be studied in the living fowl injected with a dye which is long retained in the blood stream (Chicago blue 6B). Extremely thin venules and arterioles are then seen to rise side by side toward the surface of the beautifully transparent pectoralis major, the arterioles being remarkably slender; and they so interlace, while branching, that with difficulty one perceives them to be connected with the capillaries of different small groups of muscle fibres. The capillaries appear as the finest threads, even when the blood is deep colored.

In fixed and cleared specimens having red mass in the arterial system and black in the venous, the picture is like that which would be found if the muscle were possessed of a duplicate set of vessels, with one set shifted the length of a single group of capillaries so that venule and arteriole, not two arterioles or two venules, run side by side. By teasing fixed preparations under the microscope, or rubbing them thin, the ultimate venules and arterioles can be proved to ramify in slightly different planes, the result being that while small groups of fibres have the benefit of capillaries coursing in opposite directions, this is not true of them individually, or is true only where those of neighboring capillary groups adjoin (Fig. 5). A more detailed study of the state of affairs is desirable.

Not only are the capillaries of fowl muscle extremely short but the opposite direction of flow in those lying next each other should mask the influence of any local differences in permeability.

immaterial. We have shown in a previous paper that only the possibility last mentioned explains the barring in ordinary skeletal muscle. The point to be stressed in the present connection is that the vascularization attests as definitely as in the case of pigeon muscle to special demands along the further capillary way.

DISCUSSION

An understanding of the conditions determining exchange between the blood and tissues is one of the ultimate goals of physiology; and it has attracted the attention that it deserves. In proportion as conditions have been recognized which influence exchange, reasoning upon how and where this should take place has become precise and occasionally dogmatic. There is reason to ask whether direct observation is not still a safer mode of approach to the problem presented by the process than *a priori* inference from such conditioning factors as have thus far come to light. Having observed what happens to a given substance one is certain at least that it actually passes into or out of the blood at such and such regions and in such and such quantity. In reasoning upon what should happen to it on the basis of known factors one is assuming that one knows them all, that there is none still hidden which plays a decisive rôle. It is with this point in mind that our experiments have been carried out, of necessity with substances strange to the tissues.

The observations reported here make plain the fact that in frog muscle and the mammalian diaphragm the opportunity for dyes to pass from the blood to the tissues progressively increases along the capillary way. In the frog this comes about mainly through a graded increase in permeability of the walls of these vessels, but in addition there occurs toward their further end some expansion of the surface through which exchange may take place. In the diaphragm the expansion is abrupt and considerably greater. While it will suffice to explain the especially abundant escape of substances near the diaphragmatic venules, that it plays the sole rôle in the graded increase seems unlikely, in view of the fact that permeability increases progressively along the capillaries of other mammalian muscles. Chicken muscle exhibits a significantly different state of affairs. It is supplied with what is to all intents and purposes a double circulation in closely

the transverse venule before entering it, an arrangement suggestive of that in the pigeon pectoral (Fig. 3).

The capillaries which traverse the arteriolar tree are, as in other skeletal muscles, remarkably few, a fact which attests to efficient exchange through the arteriolar wall itself (1).

So rapid is the distribution of stain to the diaphragm that no local differences are ordinarily met in rabbits sacrificed a minute or so after an injection of the highly diffusible patent blue V or brom phenol blue. The organ has then an even, diffuse, deep blue hue, even when the animal has been bled beforehand in the attempt to cut down the circulation. But if Chicago blue 6B is injected and the diaphragm inspected immediately one finds "mackerel sky" barrings of color indicative of a gradient of distribution of the dye (Fig. 6).

Into an ear vein of an etherized rabbit 3 cc. per kilo of a standard, isotonic solution of Chicago blue 6B is run in the course of 2 minutes. The carotids are immediately severed, the abdominal wall slit from symphysis pubis to ensiform, and the under side of the diaphragm inspected *in situ*. Everywhere the muscle is seen to be barred with blue. The chest is now opened along the sternum, the large vessels clamped off below and above the diaphragm, in the order mentioned or the reverse, and the organ is cut from the thorax, spread and inspected between glass plates. It shows a diffuse blue staining with superimposed bars of much deeper blue, each having a transverse collecting venule as axis. More of the tissue is involved in the bars of Chicago blue than in ordinary skeletal muscle—about one half all told. Near the arterioles the staining is neither greater nor less than elsewhere outside the barred regions. When the animal has been let live for a minute after injection the bands appear narrower because their margins are lost in a deep, general staining. Equalization of the staining is very rapid after death.

In some of our instances the abdomen was opened just prior to the injection, and the liver was seized and pulled down on one side at intervals to find whether the diaphragm contracted during the period of staining, as was regularly the case. In uninjected animals the organ showed no trace of barring, although the vascular pattern could be plainly seen when it was looked at between glass plates; and no bars appeared in animals receiving a half and half mixture of India ink and Ringer's solution in sufficient quantity to make the vessels stand out in black. Thus the possibility that the "mackerel sky" might be due in part to the vascular content was ruled out.

The question of whether the color pattern in the diaphragm is referable to a graded increase in permeability along the capillary, to increase in the amount of permeable surface, or to both combined, is

to oxygen distribution, and while commenting upon the fact that the number of open capillaries in the exercising tissue is larger than will suffice to ensure oxygen, has suggested that it points to some other need. The local elimination of carbon dioxide presents no problem in this connection because it diffuses through tissues with enormous rapidity. Determinations of the relative rate at which the metabolic products of muscle activity pass through living tissue may yield an answer to some of the questions raised by the singular capillarization of pigeon muscle. The need for research on the comparative anatomy of the muscle vessels in its relation to muscle function is an obvious one.

Despite the high blood pressure of the fowl,—averaging about 160 mm. Hg (14),—and the abundant vascularization of its muscle, very little dye gets out into this tissue as compared with the amount in mammals. The point should be emphasized in connection with this difference, though not as explaining it necessarily, that tests with dyes disclose only the further and furthest limits of vascular permeability. Schulemann has proved that the rate of vital staining corresponds nearly with the diffusibility of the dye employed (4). The most highly diffusible of our materials, phenol red, spreads through water at about the same rate as dextrose and through gelatin more than three times as slowly (1). It may pass through the capillary wall more readily than some amino acids, but without question would be found to lag far behind many substances used by the tissues; for the rate at which dextrose itself gets into and out of the vessels is almost startlingly slow as compared with that of the salts of the blood (15). Unfortunately all highly colored substances of simple constitution that we have sought to utilize have yielded an equivocal vital staining or have been toxic.

In an accompanying paper the gradient of vascular permeability will be discussed in some of its general relations. Here the fact may properly be dwelt upon that recent investigation shows the differentiation of the vascular tube to transcend far, both structurally and functionally, the conceptions of it generally held. Until a few years ago the tripartite division,—into arteries, capillaries, and veins,—carried with it a belief that the capillaries are inert organs, mere passive instruments in exchange. The demonstration of their active contractility has invested them with new meanings. The structural modifications along some of them (as *e.g.* those of nail-fold, bladder, and

spaced, short capillaries, and the blood runs in opposite directions between muscle fibres lying almost side by side. No evidence of a gradient of permeability along the capillaries has been obtainable under the conditions; perhaps none is present. In a flight muscle of the pigeon (*pectoralis major*) there exists not only the same structural provision for abundant circulation as in the chicken but an elaborate collecting system of capillary vessels.

These diverse findings bespeak the need, emphasized in a previous paper, for an equalization of opportunity where shortcomings in local maintenance would reduce the efficiency of an entire fabric. As there pointed out, the parenchymal cells of the liver compete with one another for existence (12); and the lobular capillaries converge and progressively unite on the way to the venous center, the result being that the further a cell is from the blood source the greater is the quantity of blood passing it. Thus opportunity is equalized. In the urinary bladder the capillaries enlarge along their course and interlace in such manner that any further provision to equalize the conditions along them may be unnecessary, though it is far from being ruled out. Frog skin is supplied from a capillary meshwork practically devoid of such local differences in vascular calibre and mesh size as would suffice to put the connective tissue cells in the neighborhood of the venules on a parity with their fellows closer to the arterial blood. This end is gained by a sharply mounting gradient of capillary permeability (13).

The opportunity to get rid of wastes is as important for the tissues as that to procure materials; and hence the artifices just mentioned must be examined with both purposes in view. The provision in pigeon muscle of fan-shaped aggregates of capillaries converging upon the venules would seem manifestly appropriate to some special eliminative need, though what that need one can only conjecture. Structural indications of somewhat similar character are present about the collecting venules of the mammalian diaphragm. The increase in the wall area of the capillaries of frog muscle as these near the venules may serve more for elimination than supply. So too may the remarkable permeability of the small venules of muscle generally, which is only slightly less than that of the capillaries, and in frog skin even greater (13). Krogh (2) has worked out for muscle the relation of capillary spacing

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EXPLANATION OF PLATES

PLATE 8

FIG. 1. Edge of the sartorius of *Rana pipiens*, cleared after injection of an ink-gelatin mass. The muscle has shortened somewhat with result that the vessels appear slightly contorted. Two transverse venules are seen with an arteriole between, paralleling them at a distance of about 1.3 mm. Many of the capillaries fork on the way to the venule, and cross connections become more frequent as the latter is approached. The result is an undoubted increase in vascular surface, marked in some regions, negligible in others. $\times 29$.

FIG. 2. Distribution of trypan red to the sartorius of *Rana pipiens*. The 50 gm. animal was given 0.3 cc. of the dye solution in half a minute, while unanesthetized, and decapitated $3\frac{1}{2}$ minutes later, when the muscle was dissected out and photographed between slides. Each of the transverse venules is the axis of a broad bar of stain which fades off in the direction of the arterioles. Only one of the latter is visible (the arrow points to it); but the microscope showed that each lay in the midst of unstained tissue. The general vascular arrangement is far less regular than in mammals, and so too, of course, is the distribution of the dye. The distance from venule to venule is in some cases more than 3 mm. $\times 5$.

FIG. 3. Drawing of the vascularization of an injected, teased, and cleared muscle bundle from the diaphragm. A transverse arteriole is shown with venules to either side. Few capillaries traverse the arterial tree. Those given off from it increase on the way to the venule by forking, and on nearing it some quit their course, enlarge, and run slantingly to unite with the main stem. $\times 58$.

PLATE 9

FIG. 4. Thin portion of an injected and cleared external oblique muscle of the fowl. The arteries appear paler than the veins because they contain red mass, while the veins hold black. They ramify side by side across the muscle, two veins to an artery, and break up into exceedingly fine and numerous capillaries which parallel the muscle fibres. Only the larger distributing and collecting vessels can here be made out. $\times 20$.

FIG. 5. Arrangement of the smaller vessels of chicken muscle, as shown in a teased fragment of the pectoralis major. The preparation had been treated like that of Fig. 3. The final arterioles and venules lie nearly side by side but the fact can be made out that they are in different muscle planes. They connect, not with each other but with the nearest vessel of opposite kind which lies in the same plane. The length of the capillaries is only about $\frac{1}{3}$ mm. The arrows point to artefacts which might be taken for vessels. $\times 66$.

connective tissue (16)) and the gradient of permeability along others (muscle, skin (13)) constitute evidence that the capillaries, as well as the arteries and veins, undergo differentiation along their course. The final arterioles and least venules are now known (15, 1) to share in the functions of exchange allotted in the past solely to capillaries. One may expect that as knowledge grows in the future the classification of the vessels into three categories will be supplemented by a conception of the vascular tube as a single entity which undergoes modifications that are especially various and significant along that portion of it known as the capillary.

SUMMARY

A mounting gradient of permeability exists along the capillaries of frog muscle. In chicken muscle on the other hand none has been demonstrated; but the close-knit vascularization is arranged in duplicate in such manner that the blood runs in opposite directions through the capillaries of nearly adjacent fibres. In a flight muscle of the pigeon there exists in addition to this artifice what appears to be a special collecting system of venous capillaries. In the mammalian diaphragm indications of such a system are also to be found, and a gradient of capillary permeability like that in the other skeletal muscles is probably present.

These vascular conditions are briefly considered in terms of function.

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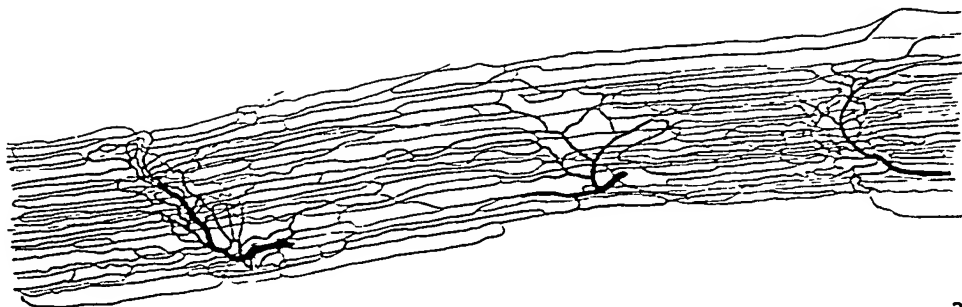
PLATE 10

FIG. 6. Half of the diaphragm of a 2000 gm. rabbit killed by cutting the carotids 9 seconds after the injection of 6.1 cc. of 8 per cent Chicago blue 6B in 2 minutes and 11 seconds. Photographed between glass plates. The "mackerel sky" barring with color in the muscular portion of the organ closely resembles that which is indicative in other mammalian muscles of a mounting gradient of permeability along the capillaries. The preparation thins into tendon at one end. Here the vessels are very sharply outlined, no stain having escaped. Natural size.

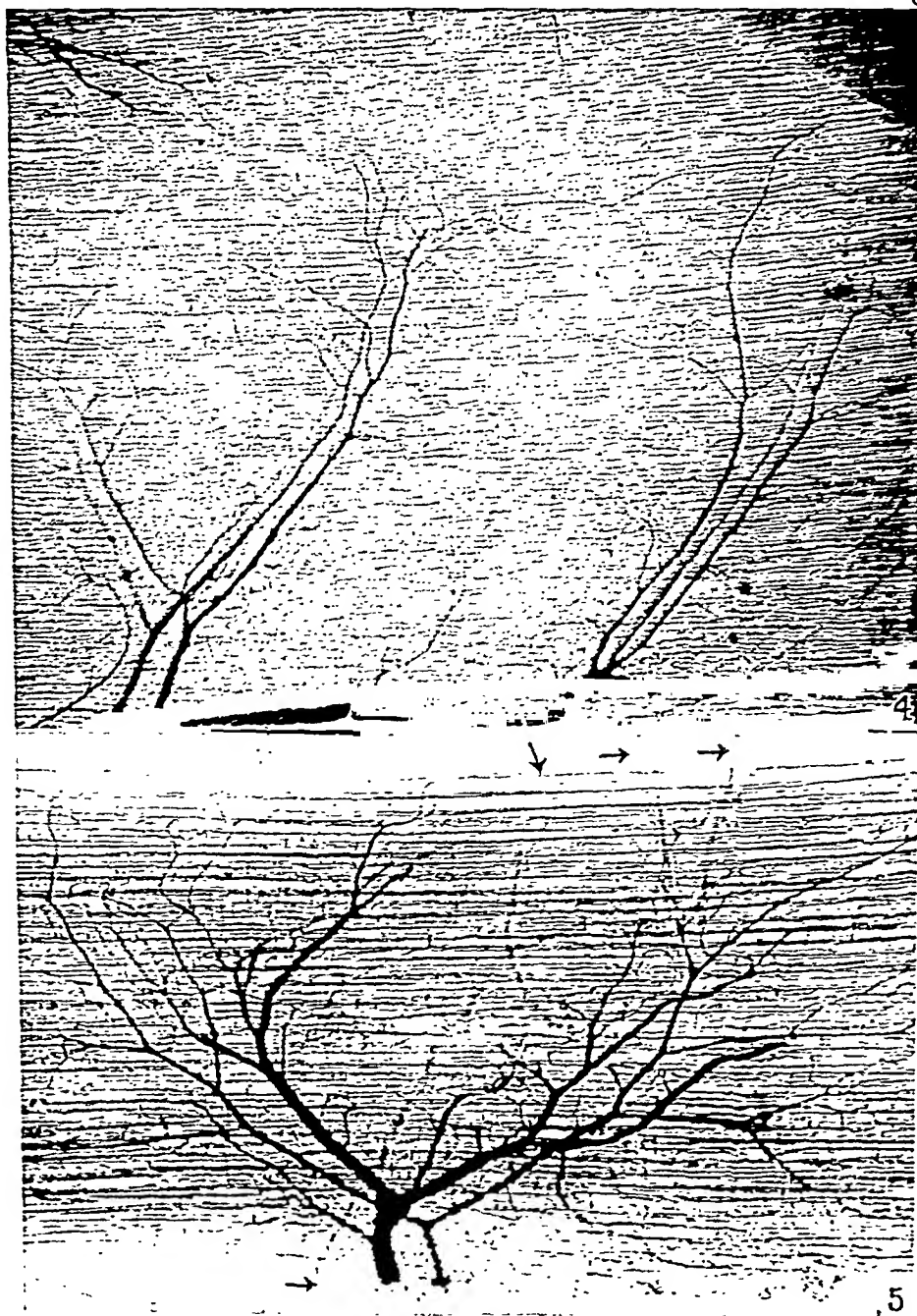
FIG. 7. The vessels in a single plane of the pectoralis major of the pigeon—from a specimen rubbed thin after injection and fixation. The transverse arterioles lie in tissue traversed by very few capillaries, whereas each venule constitutes a stem to which many such vessels converge fan-wise, after abruptly quitting their course beside the muscle fibre. The greatest distance between arteriole and venule is only about $1/5$ mm. but the length of the capillaries is sometimes more than $2/5$ mm. owing to the change in their direction. At the lower edge of the photograph the vessels appear closely crowded because two layers of them are superimposed. $\times 90$.



2



3



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6



7

but in *Rana pipiens* one sees it through a grayish mist. The compactum in both species is compact indeed, an opaque sheet with very numerous foramina, through which blood vessels, nerves, and lymphatics run vertically to reach the surface. Beneath it is a thin tela subcutanea, in which course the parent vessels. The tela is lined with endothelium on the side toward the lymph sac. The venules and arterioles emerging on the outer surface of the compactum connect with a continuous network of capillaries lying just beneath the skin epithelium (Figs. 1 and 3). It is with the escape of dye from this network that we have concerned ourselves; for deeper in the spongiosum there are no capillaries. The compactum is without blood or lymph vessels of its own (4) and it appears wholly unstained.

General Technique

The passage of dyes from the blood has been followed with the same apparatus as in previous work (1). Most of the frogs had been immobilized with curare, or etherized, or pithed; but some few have been held and studied while unanesthetized. Etherization and pithing cause the circulation to become relatively sluggish, while, furthermore, reflex movements often complicate the observations; and hence we have relied largely on curarized frogs (0.1 cc. of 1 per cent curare into a dorsal lymph sac). As soon as the animal becomes flaccid it is laid upon wet filter paper and kept moist with water, or, more frequently, it is just submerged in a shallow dish with a paraffin bottom. Adolph (5) has shown that practically no fluid exchange takes place through the water-bathed skin of normal frogs. A region is selected for observation where the capillary rete is everywhere visible, by reason of the flowing blood, with its arterial and venous relationships clearly distinguishable. The blood stands out brilliantly in light passed through a methylene blue solution, and the spread of red dyes can be best followed under such conditions. With purple and blue dyes no such aid is needed. Prior to the injection the field is sketched, and while it is watched under the binocular microscope the dye is introduced into a vena dorsalis pedis. To immobilize the foot, it is pinned through the web upon a cork sunk at the corner of the dish.

The same dyes have been used as previously (2). All were carefully purified and tested upon mice for toxicity. They were in watery solution isotonic with frog blood (that is to say with 0.7 per cent NaCl), the amount given being most often 0.1–0.2 cc. for a frog of 30 gm.; though with substances of relatively poor tinctorial qualities, and larger frogs, as much as 0.35 cc. was sometimes required. The poorly diffusible dyes were injected slowly, in the course of a minute or slightly less, and highly diffusible ones within a few seconds.

The fact that frog capillaries let through a part of the plasma proteins (3, 6) led us to try erythrolitmin, the pigment of red cabbage, and methemoglobin (of the horse). The first two proved toxic intravenously, and methemoglobin failed to pass into the tissues in distinguishable amount. The ferrocyanide method of Weed yielded uninformative pictures owing to secondary extravascular spread of the reagents.

THE GRADIENT OF VASCULAR PERMEABILITY

III. THE GRADIENT ALONG THE CAPILLARIES AND VENULES OF FROG SKIN

BY PEYTON ROUS, M.D., AND FREDERICK SMITH, M.B., B.Ch.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 11 TO 14

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In previous papers (1, 2) experiments have been described which prove that the permeability of the muscle capillaries of mammals and frogs increases progressively along these vessels, being greatest in the region where they join the venules. The present communication deals with the conditions in frog skin.

The capillaries of the frog tongue, web, and mesentery have become classical objects of study while those of the skin of the body are employed but seldom. Yet in some species the skin offers opportunities beyond those of any of the structures mentioned. To watch tongue and mesentery they must of necessity be exposed and pinned out, subjected to more or less trauma, and kept wet with a drip, which differs from frog lymph (3). In the web two skin layers come together with result in complicated vascular pictures, and the circulation is far more frequently irregular than over the body. We have judged permeability by the escape of dyes from the blood. In the web and mesentery there is almost no interstitial tissue to stain, or to serve as a ground upon which the spread of dyes can be perceived. In the skin of the body on the other hand the capillaries ramify in a transparent tissue overlying a dense, grayish white substratum against which every least escape of dye is visible.

The Vessels Studied

The skin of *Rana pipiens* and *Rana clamitans* presents the general features (Figs. 1, 2, 3, and 9) described for European frogs (4). The transparent epithelium, pierced here and there by gland outlets, covers a loose-textured stratum spongiosum of corium wherein lie pear shaped glands. In *Rana clamitans* the spongiosum is so transparent, where it lacks granule cells, that the pitted surface of the underlying "sieve layer" of the stratum compactum can be closely inspected;

became stained the vessels stood out sharply in blackish blue, and within a minute and a half, all told, the blue stain could be seen emerging, first where the capillaries entered the venules, and soon all along the further half of the capillary rete, in increasing amount as the venules were neared. Very quickly the meshes in this region became blurred with blue, the extravascular color spread laterally and became confluent, and within less than another minute the further half of the meshwork was wholly obscured by the diffuse, deep staining. While this happened the blood lost color, and in consequence the proximal portion of the capillary rete stood forth less sharply. Within 3 minutes after injection the distribution of stain from the skin vessels had to all intents and purposes ceased. The deepest color lay about the venous centers, shading off in the direction of the arterioles, rather abruptly ceasing about midway to them. In the gross the skin was now brilliantly speckled with blue dots on white. The dots were discrete, less than 1 mm. across, on a practically unstained, white ground. The fact was plain that the dye had got out so fast from the further capillary meshes that the blood was practically rid of it before any escape had taken place from the proximal ones.

These findings with trypan blue have been confirmed and extended with dyes of greater diffusibility (patent blue V, brom phenol blue, eosin, trypan red), and with the more poorly diffusible Chicago blue 6B, pontamine sky blue, and Congo red. The red dyes yield the best photographs (Figs. 4, 5, 6, 7, and 8). All escape much more rapidly from frog vessels than from those of rabbits. Phenol red, the most diffusible of our test substances, leaves frog vessels with such extraordinary swiftness, when the circulation is vigorous, that one cannot hope to perceive local differences in the rate of its distribution.

SPECIMEN PROTOCOLS

Phenol Red.—A curarized *Rana pipiens* of 23 gm. received 0.15 cc. of 2.9 per cent phenol red in 9 seconds. 1 second before the end of the injection the rete suddenly stood out in rose color,* and forthwith the dye escaped into the tissues everywhere along the vessels, the extravascular color rapidly becoming confluent by lateral spread.

Patent Blue V.—This dye is little less diffusible than phenol red (1). A *Rana pipiens* of 36 gm. was curarized and 0.1 cc. of 5.8 per cent patent blue V injected intravenously in 17 seconds. Only at the end of this time did the rete stand out in blue. The capillaries in the field drawn were somewhat larger in the neighborhood of the venules.

* The tissues of the frog are distinctly alkaline, as compared with those of mammals.

Vascularization of the Skin of the Belly of Rana pipiens

The smooth, white skin over the lower middle of the abdomen of a 30 gm. *Rana pipiens* is almost ideal for study. It is practically devoid of papillae, contains relatively few glands, and the capillary meshwork has a strikingly regular pattern (Fig. 1).

In the gross the belly skin appears shining white because of numerous "interference cells" just beneath the epithelium (Fig. 12), containing granules which reflect light. Occasionally they form an almost continuous sheet and the frog must be discarded; but usually they do not obscure the spread of dye from the vessels. While lying at the level of the capillary rete they have no evident relation to it; and they seldom encroach upon the outline of one of its meshes but serve instead to enhance its brilliancy by contrast. Occasional small areas are wholly free from them.

Venules and arterioles connect with the capillary network separately at fairly regular intervals. The meshes of the latter converge to the venules as in the liver, but they radiate correspondingly from the arterioles as well. The latter open directly into the rete, the result being that one sees the blood spray out in all directions, as from unseen fountains, in continuous non-pulsatile streams which pursue a zig-zag, helter-skelter course in the meshwork until they reach the venules. Some few of these are incorporated in the rete and course for a little distance at its level, receiving capillaries until they dip from sight (Fig. 1). The emergent arterioles are definitely narrower than the capillaries they feed. In vigorous frogs the blood current lags but little until the venules are reached.

The distance from venule to venule is usually 1 to $1\frac{1}{2}$ mm., and from arteriole to venule somewhat more than half this distance. The lozenge-shaped meshes of the capillary network measure $\frac{1}{16}$ to $\frac{1}{8}$ mm. in greatest diameter.

The capillaries appear larger near the vein. This is mostly the result of the brighter hue of the blood as it becomes oxygenated during its progress within the rete (7). The influence of the factor can be readily appraised by injecting a dye which stains the plasma deeply and does not soon escape from it. Then the rete stands out like a drawing in colored ink and one perceives that the local differences in calibre are exceedingly slight, though some capillaries become a little wider near the venules (Fig. 1). It is not difficult to find regions where the difference is negligible, and ordinarily we have chosen such for study. Marked enlargement of the blood channels begins with the venules.

The Escape of Dyes into the Skin of the Belly

Our first observations were made in late April upon pithed or curarized frogs.

A 4 per cent trypan blue solution in twice distilled water was injected intravenously, 0.2 cc. for a frog of 23-31 gm., in 45 seconds to $1\frac{1}{2}$ minutes. As the blood

curarized *Rana pipiens* of 34 gm. was injected with 0.2 cc. of 10 per cent Congo red in 1 minute and 6 seconds. The rete stood out in deep red after 22 seconds, but only after 5 minutes was extravascular dye noted, first in the tissue around the superficial venules, and after another minute about the adjoining capillary meshes. At the end of 13 minutes the staining was still pale and localized, none having occurred along the proximal half of the rete. The circulation was now sluggish, with contraction and emptying of some of the capillaries. Most of the dye had been lost from the blood, the integument of the thighs being deeply stained.

In numerous other experiments like phenomena were observed. Always, save in the case of phenol red, one could perceive that the rate at which dye escaped into the tissues, as well as the quantity, increased progressively along the capillary way from arteriole to superficial venule, reaching a maximum about the latter. Poorly diffusible dyes passed into the skin only from the venules and the adjoining capillary meshes during the considerable period before the blood was depleted of them.

Periglandular Staining

A distinctively great permeability of the capillaries which encircle the skin glands was noted frequently under conditions which suggested that functional activity might be its determining cause. The staining calls for description because of the complication it offered. Its physiological implications will be dealt with in a later paper.

The glands of the abdomen of *Rana pipiens* are pear-shaped structures in the stratum spongiosum (Figs. 11 and 13). Most are surrounded at the neck by a mesh of the capillary rete which differs from the others in being circular, slightly smaller, and with a somewhat narrower vascular lumen (Figs. 1 and 6). No glands are found immediately next the emergent arterioles or the superficial venules, but they are scattered throughout the rete elsewhere in no evident relation to the vascular pattern as a whole. Nearly all are of one sort (mucous glands). They consist of a single layer of epithelium which changes in appearance with the secretory changes, as in European frogs. In our first experiments, conducted in the early spring upon animals that had been kept in the ice box, periglandular

water, precipitated by the gradual addition of concentrated hydrochloric acid, washed on a filter paper, dissolved again by the very gradual addition of sodium hydroxide solution, using the least amount necessary for the purpose, and allowed to crystallize out. A 2 per cent solution of the material thus prepared had a Δ of 0.075°C.

32-42 sec.—The dye can be seen to pass out from the venules of the rete and the adjoining portion of the capillaries, from the venules first and most intensely. 1 min. 9 sec.—The stain has now escaped everywhere along the capillary network, grading off in the direction of the arterioles. Round where they join the rete the hue is notably pale; but the extravascular dye is spreading rapidly. In the pale regions "interference cells" are no more frequent than elsewhere. 2 min. 11 sec.—Circulation continues excellent. The rete can still be seen in the region of the arterioles, but everywhere further on it is blurred by stain. 2 min. 20 sec.—The coloration has become diffuse; local differences are no longer perceptible.

Brom phenol blue diffuses more slowly than patent blue but far faster than the trypan dyes. A *Rana pipiens* of 26 gm. was curarized and a spot selected for observation where the capillary meshes were everywhere of the same calibre. "Interference cells" were unusually few. An injection of 0.2 cc. of 2.7 per cent brom phenol blue was given in 29½ seconds.

Stained blood outlined the rete within the first 14 seconds; after 27 seconds the dye was observed to be coming out from the further portion of the network; and in another second from it everywhere. After 35 seconds altogether the dye was escaping abundantly along the entire course of the vessels, though still in greatest quantity near the venules. After 45 seconds the network was no longer visible, so deep was the coloration—a brilliant, dark, purply blue. The swift circulation could be discriminated with difficulty. After 2 minutes and 55 seconds the skin staining had become general.

Eosin.—A curarized *Rana pipiens* of 28 gm. was injected in 22 seconds with 0.25 cc. of 4.3 per cent eosin. The blood flow did not slacken locally, yet the rete only became outlined in color after 1 minute and 24 seconds in all. 25 seconds later the stain had appeared in the tissue about the further capillary meshwork. After 3 minutes in all the plasma was so far decolorized that moving corpuscles could be seen. There was still no staining round where the arterioles gave into the network, though elsewhere the color had spread laterally and was becoming diffuse. The hue was most intense where the collecting venules dipped down. After 4 minutes and 15 seconds the staining was diffuse, though still with pale spots where arteriolar blood entered the network. After 5 minutes and 15 seconds the skin was brilliantly and evenly red.

Trypan Red and *Chicago Blue 6B*.—The work with these substances finds place further on.

Congo red is the most indiffusible dye that has yielded positive findings.* A

* The difficulties of ascertaining the molecular weight of Congo red have become classical (Freundlich, H., *Kapillarchemie*, Akademische Verlagsgesellschaft, Leipzig, 1923, 766). The results of freezing point determinations upon solutions of the dye in water give almost no indication of what its state will be when in plasma. There is much in the literature to suggest that the same holds true of many other dyes, if to a less extent.

The Congo red of the present work (Grübler, Dr. K. Hollborn) was dissolved in

The escape of highly diffusible dyes takes place so quickly into the skin of the upper, inner surface of the thighs that with them there is not time for local differences of distribution to be perceptible. However, with Chicago blue and Congo red a progressively increasing escape along the capillary way is readily to be noted. With the highly indiffusible Congo red it is attested in the gross by a mottling of ruddy dots on a white ground, each dot with a venule at its center.

The Skin Circulation of Rana clamitans

The rete on the abdomen of *Rana clamitans* is not plainly to be seen. On the upper, under surface of the thighs of females, however, there are patches like pellucid lagoons in a skin tinselled for the rest with "interference cells" or blackened by pigment cells. One peers into these lagoons as into water and sees the blood hurrying through the narrow meshes of a network of vessels that are themselves invisible. An especially large area of the sort is usually to be found just below the groin.

Through the skin the stratum compactum lies bare to view as an opaque, light gray sheet pitted by numerous funnel shaped orifices through some of which the vessels rise or disappear, not nearly filling them. Injections of gelatin-carminé and India ink masses into blood and lymph vessels, respectively, show most of the foramina to be pierced by a lymphatic only, the proportion with a blood vessel being the same as during life in warm weather. The compactum is often ridged slightly where the larger vessels run beneath it in the tela, and looking along the line of these ridges in the living frog one sees arterioles emerging at intervals or venules dipping out of sight. They pierce the compactum separately, as in European frogs (4). Only once have we seen venule and arteriole occupying the same foramen.

The arrangement of the superficial capillary network differs greatly from that in *pipiens* (Figs. 2 and 9). The meshes are larger, elongated or lozenge-shaped, 1/8 to 1/4 mm. in width, by several times this length often. Each encloses one or more skin glands, a fact which becomes evident only after introduction of a dye. There is less radiating from arterioles and converging upon venules than in *pipiens*, these vessels joining the network at very irregular intervals, often several of the same sort close together (Fig. 9). The blood may follow a tortuous course in the network or may flow almost straight, according to local conditions, the distance traversed to the venule being seldom as much as 1 mm., often far less because an arteriole emerges and venule dives down through adjacent foramina. Near the arterioles flow is relatively brisk. Not a few venules run for a little distance as part of the network, growing larger as new capillaries enter them, while others turn abruptly down. Those first mentioned may become three times as wide as capillaries while still a part of the rete. Under the circumstances of our experi-

staining did not attract attention. But in the later work, carried on in an especially warm May and June with frogs that had lived at room temperature, it frequently dominated the picture. Many individuals were still encountered, however, in which this was not the case. In frogs showing a special staining about the glands the outline of some or all of the encircling capillary rings became blurred by escaping dye before any could be seen elsewhere (Fig. 5); and the color, rapidly becoming confluent, gave rise to irregular patchings (Fig. 6) which in the gross differed strikingly from the ordinary dotting with color. The gland itself seldom stained, but was distinguishable by a collar of dye. Even with phenol red a special periglandular escape of dye was not infrequently perceptible during the instant before the skin flushed rose everywhere; and with poorly diffusible substances (Chicago blue 6B and Congo red) sometimes the only coloration prior to effective depletion of the blood occurred from the gland circlets. In not a few experiments the periglandular escape, like that from the meshwork generally, took place first and was greatest in the further capillary region.

Numerous tests have been made, with Chicago blue, Congo red, and trypan blue, upon the influence of the circulating amount of dye on periglandular staining. These leave no doubt that the smaller this amount the more marked is such staining as compared with that of the skin generally. For example, with large doses of Chicago blue 6B in frogs submerged at 16°C. we observed not merely a staining from the capillaries about glands but everywhere from the vessels of the further portion of the capillary rete, whereas with a small dose the only extravascular coloration was about the glands.

The experiments on periglandular staining illustrate a rule of practical importance in the study of regions where vascular permeability is relatively poor, as it is in the muscles, namely that a great deal of dye must be put in circulation, or one very highly diffusible, else all will get out of the blood into certain organs or regions before any has escaped elsewhere.

The Escape into the Skin of the Thighs

Near the crotch the thigh skin of *Rana pipiens* is rugose, with large papillae. Most of the arterial capillaries emerge at the summit of these latter—where “interference cells” are often so many as to mask any staining; and the blood streams in a close-meshed rete down their sides to reach venules that dip under in the valleys. Here and there, however, the situation is reversed, with result in an opportunity for enlightening comparisons. Under such circumstances the arterial capillaries emerge in the valley and the blood mounts the side of a papilla to vanish into a venule near its summit. Generally speaking the width of the capillaries increases considerably as the venule is neared.

In this experiment the phenol red was isotonic with mammalian blood; but its amount was so small that the hypertonicity for the frog could be neglected.

Patent Blue V.—A curarized *Rana clamitans* of 40 gm. was injected in 10 seconds with 0.15 cc. of 6 per cent patent blue V. The rete stood forth in blue, and within 25 seconds the dye could be seen emerging from venules and venous capillaries. After 50 seconds the tissue supplied from the proximal (arteriolar) half of the meshwork was alone free from stain; and after 2 minutes it was coming out here also, but in slighter quantity. A graded increase in intensity and amount of the staining now existed all along the capillary way. After $2\frac{1}{2}$ minutes the whole spongiosum was diffusely blue because of secondary distribution of the dye, yet the regions where arterial blood entered the capillary web were paler than elsewhere. The glands were now plainly outlined because unstained.

4 min. 28 sec.—The diffuse coloration remains more intense in the further portion of the capillary rete. The color is deepest where the veins dip down. 5 min. 8 sec.—Circulation continues excellent. 6 min. 50 sec.—The whole spongiosum is like a blue jelly, least tinted around the emergent vessels.

Trypan Blue.—A curarized *Rana clamitans* of 62 gm. was injected with 0.3 cc. of 2.7 per cent trypan blue in 1 minute and 13 seconds. At the end of the first 38 seconds the rete was brilliantly blue, but not until after 2 minutes and 25 seconds in all did escaping dye begin to blur the vessels, first the superficial venules, then the neighboring meshes of the rete. At the end of 4 minutes and 30 seconds extravascular trypan blue was present everywhere along the latter except in the immediate neighborhood of the arterioles. Like other dyes it was seen to escape from the blood as a spreading fuzz of color, broadest and most pronounced next the superficial venules, thence gradually narrowing and paling in the direction of the arterioles.

5 min. 52 sec.—Still no staining where the arterioles join the rete. 6 min. 57 sec.—The dye has almost all left the blood, which is circulating briskly. 8 min. 12 sec.—Wherever one finds no staining in the region where a vessel sends blood into the rete, that vessel can be identified as an arteriole.

Chicago Blue 6B.—A curarized *Rana clamitans* of 56 gm. was injected with 0.3 cc. of 5.2 per cent Chicago blue 6B in the course of 1 minute and 5 seconds. After 28 seconds the vascular rete blued, and within 1 minute and 28 seconds the blue could be seen passing out into the tissue next the superficial venules, and in a few more seconds into that about the neighboring capillary meshes. During the first 2 minutes no spread took place from the portion of the rete nearer the arteriole.

3 min. 25 sec.—The dye has now passed out all along the capillary way except from those meshes into which the arteriolar blood directly pours, and it has obscured much of the network. The tissue supplied from the meshes nearest the arterioles attracts attention by reason of its lack of color. 8 min.—The picture is essentially unchanged. 11 min. 50 sec.—Still no staining where the arterioles join the network. A slow, secondary spread of color through the tissue is taking place. The glands begin to show brilliantly because unstained in a stained matrix.

ments, the entire capillary network seemed to be utilized by the blood stream. We never observed new channels to open even when considerable quantities of fluid had been given intravenously.

From this description it is plain that the almost diagrammatic distribution of dyes occurring in *pipiens* cannot be expected in *clamitans*. Nor is it found. But on the other hand the size of the vascular meshes and the transparency of the tissues enable one to watch closely the escape of dyes along individual channels. The course of flow shifts suddenly at times, but far less often than might be expected from the many pathways. Occasionally a circulation round and round a single mesh is observed, owing to tangential entrance of the current of blood. It is easy to select for observation capillary channels of the same width all the way to the venule (Fig. 9), and thus to rule out changes in area of permeable wall as affecting dye distribution. We have ordinarily chosen fields where the distance to be covered between arteriole and venule was a long one.

Escape of Dyes into the Skin of Rana clamitans

The findings in *Rana pipiens* have been confirmed and extended in *Rana clamitans*. In *clamitans* staining takes place somewhat the more slowly, a gradient of distribution being demonstrable even with phenol red. Only very exceptionally does a special escape of dye occur about individual glands.

SPECIMEN PROTOCOLS

Phenol Red.—A curarized *Rana clamitans* of 60 gm. was injected with 0.3 cc. of 4 per cent phenol red solution in 35 seconds. Several emergent arterioles were visible in the region selected and drawn, with distant venules that collected the blood from a capillary rete having no intermediate connections with the underlying tissue.

Within 16 seconds after the injection was begun the rete stood out brilliantly in red. After 52 seconds extravascular dye was noted around the surface venules, and in another 16 seconds about the neighboring meshes of the capillary rete itself. The circulation continued rapid. At the end of 2 minutes and 8 seconds in all, the dye was spreading laterally from next the vessels. After 2 minutes and 20 seconds some was present outside the walls of the capillary network everywhere, but the intensest staining was still about the venules, the color grading off in the direction of the arterioles. Even after 2 minutes and 38 seconds no diffusion had occurred around the latter. After 4 minutes and 12 seconds a general red staining had nearly obscured the vessels, yet after 7 minutes and 56 seconds the fact could still be ascertained that wherever there was a deeper patch of color a venule lay in its midst, and wherever an especially pale one arteriolar capillaries. The frog was now brilliant red wherever the corium could be seen.

venules, and then from the neighboring capillary meshes in an abundance that lessened progressively in the direction of the arterioles. The regions where these vessels joined the rete remained completely unstained. Similar findings were obtained in *Rana pipiens*, the distribution being even more regular (Figs. 4, 7, and 8). With large amounts of dye local differences in the staining were emphasized and the amount of unstained tissue reduced. With small amounts on the other hand, the coloration was an even one, for reasons already dealt with (1).

The findings were so contrary to those of Landis' that we have repeated his observations upon the mesentery.

This was done upon *Rana pipiens*, a species Landis employed. We have made several important deviations from his technique, while carefully following it in other respects. One was the utilization of isotonic dye solution, another the injection of the dye immediately after the field of study had been exposed and hastily sketched. The field was irrigated with oxygenated Ringer's solution at pH 8.2, and all animals were discarded in which the local circulation was not brisk. Landis did not inject the dye until after the mesentery had been in contact with Ringer's for an hour or two; and he noted that then the small vessels were in various stages of dilatation and secondary constriction. In view of his further observation that dyes pass with extreme rapidity through the capillary walls of dead or moribund animals such a wait seems hazardous. Landis observed the course of events along a single capillary whereas we have noted it everywhere in a low power field. Special care was taken to avoid pressure on the mesenteric veins.

Not only trypan red, but brom phenol blue, trypan blue, and Chicago blue 6B have been used. The findings were consistent. Save in some enlightening special instances, to be gone into later, the dyes have regularly escaped first from the distal portion of the capillaries and the smallest venules. Often none was seen to get out anywhere else. The extravascular dye was not washed away by the Ringer's as it emerged but was visible within the mesentery as a brilliant fuzz of color about the vessels, which spread away from these, not along them.

The escape of stain in rapid and disorderly fashion from isolated capillary segments was frequently noted; and when the mesentery had been irrigated for an hour prior to the injection this was especially frequent. The vascular conditions were then obviously abnormal. In an animal injected with brom phenol blue,—which escapes into the tissues with great ease,—a dense, blue staining took place from the arteriolar capillaries as these were entered and so much dye passed out here that the blood further on was never more than pale blue. Under such circumstances there was no chance for the usual gradient of distribution to assert itself. In other instances, as well, an escape of brom phenol blue took place from the proximal portion of the capillaries as the dye reached them but that from their further regions soon came to predominate.

The dyes regularly appeared first and in greatest quantity outside those collecting venules which formed part of the superficial vascular network, and later and in less amount from its capillary meshes. In the case of highly diffusible dyes staining occurred from the meshwork everywhere, though with progressively increasing rapidity and intensity along the way from arteriole to venule. The more indiffusible the dye the more did its escape tend to be limited to the venule and the furthest capillary region, and the slower was its spread both from the blood and through the tissues secondarily.

The Staining with Trypan Red

Landis has studied the escape of trypan red (vital red HR) from individual capillaries of the frog mesentery (8). He states that during the initial period of staining the dye frequently "did not come out equally along the entire length of any one capillary, but in greater amount outside the arterial portion of the vessel." Because of his findings we have made many tests with trypan red, which is excellently tolerated. Landis injected a saturated solution (quantity not mentioned). We find that 8.25 per cent of the pure Grubler preparation (Dr. Karl Hollborn) provides a saturated solution at room temperature, but that 2.8 per cent is isotonic with frog blood. A 2.8 per cent solution has been used in our work.

When 0.25 cc. of a 2.8 per cent solution is injected in the course of about a minute into a curarized 50 gm. *Rana clamitans*, the capillary rete stands out brilliantly; and by the time the injection is ended escape of the dye has begun. Always, when the circulation is brisk, it can be seen to emerge first from the collecting venules incorporated in the rete, and after a few seconds more from the further portion of the capillary meshwork everywhere, but in gradually lessening quantity toward the arterioles. The staining in the further capillary region becomes intense and confluent after a few minutes owing to lateral spread from the meshes, but round about where the arteriolar blood enters the network the tissue is still wholly devoid of color. The plasma has now paled so greatly that no further escape of dye can be expected here. Nor does it occur. For a long while the meshes receiving arterial blood can be discriminated by the pallor of the tissue in which they are situated. Even after an hour the skin is still stippled in the gross with red dots where lie those meshes adjacent to a venule.

The rate of this succession of events varied in the individual case. The dye escaped in a ragged fuzz or fringe, first about the superficial

portions of some capillaries and a little later escaping in a blue fringe here and there irregularly from certain segments only of these latter. Soon though, escape in the further capillary region preponderated over that elsewhere, and the colored barring developed which is characteristic of the gradient of distribution.

Influence of the Local Vascular State

On the way from skin arteriole to venule the blood passes through sections of a greater or less number of the meshes which make up the superficial rete. One can readily see in *Rana clamitans*, especially after the injection of a poorly diffusible dye, that some portions of the individual capillary mesh are not infrequently narrow whereas others are broad. Such dyes often escape from the broad portion of the mesh before they do from the narrow, although the blood flows briskly through both. This fact could sometimes be discerned even with trypan blue and trypan red, it frequently could with Chicago blue 6B, and most often and best with pontamine sky blue. The passage outwards of this last was limited almost entirely to the superficial venules and the adjoining meshes of the capillary network during the 15 or more minutes following its injection, and it spread very slowly from next these vessels; but the pattern was frequently confused by a deep staining here and there from capillary segments of distinctively large calibre.

Occasionally in *Rana clamitans* one saw dye passing out from certain portions of the linkage of meshes constituting the vascular way, and not from others, irrespective of their place with relation to arteriole and venule, and despite an active blood flow through them all, and a generally even calibre. The interpretation of such instances cannot be attempted.

All in all, the rule seemed to hold that wherever the capillary was wider, other things being equal, more dye escaped through its wall. There existed, of course, more surface to escape through, while furthermore dilatation is known to render capillaries especially permeable (10). The distribution of poorly diffusible dyes was far more evidently conditioned by local vascular differences than that of highly diffusible ones.

The arrangement of the mesenteric capillaries is so irregular that in experiments such as the foregoing one could not hope to obtain a gross color pattern like that which in muscle and skin provides statistical evidence of a gradient of distribution along the capillaries; but the gradient was so marked that its existence was readily discernible under the microscope. From the venules the dyes seemed to get out a little more slowly than from the adjoining portions of the capillaries.

A relatively abundant escape from the arteriolar region of the capillaries was always traceable either to great diffusibility of the dye or to a damaged capillary system. Landis states that secondarily there occurred in his preparations "a rapid vital staining of the connective tissue around the smaller venous capillaries and particularly the smallest venules." Since this did not happen until 10 minutes after the introduction of dye, its relation to the gradient of distribution that we have studied is problematic. In view of Conklin's recent demonstration (9) that frog capillaries flushed with Ringer's solution become abnormally permeable, it has seemed unnecessary to repeat Landis' perfusion experiments with Ringer's containing various dyes. Some irregular phenomena observed during the study of the skin have pertinence at this juncture.

In a thin *Rana pipiens* of 35 gm., the circulation over the belly was sluggish and irregular, with areas of stasis here and there. 0.25 cc. of trypan red solution was injected. It appeared in the tissues around some arterioles giving into the rete before the stained blood had had time to traverse its meshes, but after this had happened dye got out very rapidly from its further portion, especially from the venules, and by the end of 2½ minutes the characteristic, graded staining indicative of the gradient had developed. Here and there, however, were small, persisting patches of deeper red, testifying to an especially abundant escape of dye in certain arteriolar regions.

After the injection of eosin into a *Rana clamitans* with good circulation the dye was seen to escape from a few of the emergent arterioles and from the neighboring portions of the capillary meshwork when none had as yet passed out further on, though stained blood had reached the venules. The dye came out from one side of some vessels only, like an ecchymosis.

Similar abnormalities were sometimes encountered in the staining of frog muscle (2).

In a *Rana pipiens* having but a sluggish flow through the muscle the blood stained with pontamine sky blue could be seen to enter and pass through the exposed sartorius with extreme slowness, getting out as it did so from the arteriolar

shoulders. Here an occasional arteriole with a definite muscle layer has been observed in the spongiosum. The other vessels of this layer appear to be walled only by endothelium.

Scrutiny of the Findings

Do the dyes actually escape where first seen? Direct observation leaves no doubt of this. When an intense stain of appropriate diffusibility has been thrown into circulation one can watch it extend into the tissue from the blood of the least venules and the neighboring capillary meshes, at first emphasizing and broadening their outline (Figs. 4, 5, and 8) and then encasing them in a brilliant fringe or fuzzy sheath of color which, deepening, soon obscures them. Meanwhile no dye leaves the blood in the proximal capillary region. Its secondary, extravascular spread, often very rapid, is not along the course of the vessel but away from it. All this can be noted especially well in the pellucid skin of *Rana clamitans*.

As in mammals the local distribution of color depends to no slight extent upon the quantity of dye (1). When but little is brought by the blood the distribution taking place from the capillaries is an even one. Inequalities develop only when so much is brought, or a dye so indiffusible, that not enough is lost by the way to compensate for those influences which make for progressively greater staining as the blood advances along the capillary channel. When a highly diffusible dye is introduced into mammals a general staining rapidly ensues in the muscle, with an intenser staining superimposed thereon in the further capillary region. We have only exceptionally noted this in frog skin. The local color differences developing in it are so pronounced as to indicate that the opportunity for escape of the dyes must increase very greatly along the capillary.

There can be no doubt that many of the capillaries widen slightly as the venule is approached, with result in an increase in the surface through which exchange may take place. As already mentioned, we ordinarily selected for study regions in which the capillaries were of approximately the same size all along. Yet may not a generalized dilatation of the further portion of these vessels, occurring after the dye injection, have been responsible for the greater escape in this region? Scarcely. For the stained blood caused the vascular net-

The Structure of the Skin

The tissue into which our dyes escaped has a simple histology.

The skin of *Rana clamitans* (Figs. 10, 14, and 15) is considerably less than 250μ in depth. The vessels coursing in the thin tela subcutanea pierce the stratum compactum perpendicularly. This dense and relatively thick layer of corium contains few cells. The spongiosum and epithelium overlying it are together only between 50 and 80μ deep in the regions where the escape of dyes is best watched. The epithelium occupies about 30μ of this total. The glands lying in the spongiosum are simple, flask shaped structures, with their bases in shallow depressions in the compactum. The spongiosum itself is a very loose connective tissue containing many star and spindle shaped cells and fibrillae.

The arterioles piercing the compactum to join the capillary rete have walls but one cell thick in cross-section (Figs. 14 and 15), like the capillary rete itself. Injections under pressure of carmine mass or India ink into a subcutaneous lymph sac of the leg disclose an abundant, closely anastomosing meshwork of broad lymphatics immediately beneath the rete, and paralleling it. The channels appear huge as compared with those for the blood, and their pattern has no evident relation to that of the capillary rete. Each of the foramina in the stratum compactum, including those occupied by a blood vessel, contains a lymphatic connecting with the meshwork. Langer long ago noted all this of European frogs (11).

The skin of the abdomen of *Rana pipiens* (Figs. 11, 12, and 13) is thicker, sometimes measuring 350μ , and it has a thicker epithelium and glands far more widely separated. The spongiosum is from 60 – 120μ deep, on the average about 80μ . The layer of "interference cells" is situated at the same level as the capillary rete (Fig. 13), and the escape of dyes must be followed in regions where these cells are scattered evenly, else erroneous inferences may be drawn, since they do not stain. Where they are numerous the region remains white. The glands are of two sorts, ordinary mucous glands and "granule" glands, the latter sometimes as much as $1\frac{1}{2}$ mm. or more apart. The flask-shaped mucous glands are fairly numerous. They occupy the superficial regions of the spongiosum. The connective tissue of the spongiosum is loose and has the same general make-up as in *clamitans*, and the stratum compactum shows but a poorly defined "sieve layer." The tela subcutanea is thicker than in *clamitans*, with a dense stratum of "interference cells," and the walls of its arteries and veins show a muscle layer. This extends to the arterioles of the compactum, but the venules piercing the latter show only endothelium, like the superficial collecting venules with which they connect. The arterioles emerging into the spongiosum appear, in our preparations, to be walled by a single layer of cells only; but we have made no effort to demonstrate Rouget cells.

On the inner side of the thigh of *pipiens* near the crotch the skin is raised into papillae, the epithelium is thicker than on the abdomen, and large glands sit side by side, with the capillary rete running in a close, regular meshwork over their

up, with result that on entering the cutaneous veins it has the lowest CO_2 content to be found anywhere in the body (12). The course of events along the capillary way is in this respect the precise opposite of that in muscle. Since the gradient of permeability is similar in both tissues the conclusion seems justified that neither changes in the CO_2 content of the blood nor those of pH (as determined by CO_2) can be the cause of the gradient. Nor can changes in the oxygen content, since O_2 is taken up along the cutaneous capillaries, whereas it is given off along those of muscle.

The veins draining both the muscles and the portion of skin that we have studied in the frog connect with a portal system, and in consequence the fall in pressure along the capillaries is probably far less than in mammalian muscle, where resistance to flow is very marked (13). It may be recalled in this connection that dyes escape from the superficial cutaneous venules of frogs even more rapidly than from the adjoining capillaries, though from those of frog muscle a little the more slowly (2).

The highly permeable venules of the frog skin form part of a rete made up for the rest of capillaries. Their distance from the nearest capillary is the same as that from capillary to capillary, showing that they serve the tissue as do these latter. There is anatomical evidence that venules largely take the place of capillaries in the skin of man (13) and the mouse (14), but that in mammalian muscle they are somewhat little less effective than these vessels (1). In the pectoralis major of the pigeon and the diaphragm of the rabbit the capillaries enlarge toward their end and converge upon the transverse venule in so singular an arrangement as to suggest that special, eliminative tasks are laid upon them (2). Fröhlich and Zak (15) have witnessed an early escape of indigo carmine and potassium ferrocyanide in certain regions along the veins draining the frog's tongue; but the phenomenon was pronounced only when this member had been pulled from the mouth and pinned flat. They traced it to the local obstruction offered by valves to the transmission of back pressure when the veins nearer the heart contracted. The drawings of Fröhlich and Zak show the staining to have no resemblance whatever to the orderly escape of dyes that we have witnessed.

What can be the cause for the progressively increasing escape of dyes along the capillary way? Is it explainable in terms of the factors recognized as principally conditioning exchange with the tissues, namely hydrostatic pressure, osmosis, and diffusion? The possibility that these, severally or together, are responsible for the gradient will now be taken up.

work to stand out so sharply that any abnormal dilatation should have been clearly visible for a greater or less period; and none was seen. The influence of dilatation has been completely excluded by experiments with frogs submerged in water at 16°C. for comparative observations on periglandular staining as affected by dye quantity. The incidental cooling caused a contraction of all the skin capillaries with result that after the dye injection the rete appeared extremely narrow and of even calibre all along, the only vessels larger than the rest being venules. The circulation remained exceedingly brisk, and the color pattern due to the escape of dye differed no whit from the usual.

When curarized frogs are submerged in ice water the skin circulation ceases to all intents and purposes for a few minutes, the only visible flow being a dribble through the superficial venules. The amount of dye passing through the individual capillary under such circumstances is negligible; yet there is a constant if slow flow of it along the veins. Under these circumstances one might expect the tissue next the latter structures alone to stain, as actually happens.

Not improbably some of the dyes had an affinity for certain skin elements, though none came to attention during the experiments. We used many dyes in order to minimize the peculiarities of any one as a factor in the findings as a whole. One would have expected specific affinity to manifest itself most markedly by a color pattern when but little dye was given; yet only under these circumstances was the observed staining an even one. The spread from the vessels into the loose spongiosum as a fuzz of color did not in the least suggest a selective staining of cells and fibrils but instead an extension into the fluid of intercellular spaces.

DISCUSSION

The findings in frog skin accord with those in frog muscle (1) and in mammalian muscle as well (2). A mounting gradient of permeability exists along the capillaries of all three. Its presence in frog skin is the more enlightening because of local conditions which offer a check upon the influence of some that obtain in mammals.

The skin of the side and belly of the frog receives highly venous blood from a branch of the arteria pulmo-cutanea, and as this flows through the capillary rete it gives off carbon dioxide instead of taking the gas

affinities the more diffusible a dye proves *in vitro* the more rapid is vital staining with it. Not only is the rate of coloration dependent upon the diffusibility of the coloring matter but, in our experience, the proportion of the capillary from which its escape takes place. Escape is regularly greatest, however, in the very region where the concentration in the plasma is least, owing to loss to the tissues along the capillary way.

Some of our test substances, as *e.g.* patent blue V, pass through the capillary wall with such swiftness that their osmotic influence, as affecting exchange with the tissues, must be considered negligible. Others, like Congo red, which might have exerted a significant influence, were introduced in isotonic solution, as was routine for that matter. According to the classical view the only osmotic influence worthy of note as determining normal exchange with the tissues is that of the blood colloids, which should attract fluid into the vessels in greater and greater proportion as the opposing force of hydrostatic pressure becomes less on the way to the veins. This osmotic influence should act, not to promote the passage of dyes into the tissues in greater and greater amount as the blood progresses along the capillary, but increasingly for a retention of these substances.

It is plain that if hydrostatic pressure, diffusion, and osmosis determined the local phenomena along the capillary way these should be precisely the opposite of the ones observed. In a previous paper we have described experiments which indicate the presence along the capillary of some stable arrangement which controls permeability (1). It may be that the perivascular tissue acts as a barrier about the capillary and becomes increasingly loose-textured along this vessel with result in an easier spread of dyes. Local differences in the vascular wall itself seem a more likely possibility. Differentiations of shape along certain capillaries have already attracted attention, as *e.g.* along those of the nail-fold. Sandison has recently reported (18) of capillaries new-formed in proliferating connective tissue of the rabbit's ear that toward the vein the cells are flatter, the channels wider, the current slower. Subtle modifications in structure may well be responsible for the gradient of permeability along the vessels that we have dealt with.

Hydrostatic pressure drives the dye-laden blood along the capillaries, and, other things being equal, the amount that passes a given surface during a given period will depend on the local pressure differences,—all of which is to say that the rapidity with which vital staining occurs cannot but be conditioned in the large by the pressure factor. The view has been strongly held that substances tend to pass from the blood to the tissues from the first portion of the capillaries because the pressure is highest there, and from tissues to blood further on where the osmotic influence of the intravascular colloids supposedly becomes the dominant force. In a preceding paper we have dealt with this view in so far as it concerns the gradient along mammalian capillaries, and have shown that it is incompatible with the facts (1). In the case of the frog, however, the hypothesis has special claims to attention since the capillaries of this animal are far more permeable than mammalian ones. Lymph formation is an especially active process in the skin, where even a part of the proteins leave the blood (3, 16). There must be a continual, rapid passage of water from the vessels; and the question of where and how this passage takes place is highly relevant to the problem of dye escape, since the dyes are in watery solution. According to Landis (8), water filters from frog blood where the hydrostatic pressure is greatest, that is to say along the first part of the capillaries, and it carries dyes with it, the result being that both the region and the rate of their escape into the tissue are directly determined by the pressure factor. Our repetition of Landis' observations on the mesentery do not support this view; for we found that the nearer conditions approximated the normal the more regularly was dye escape greatest where it should have been least if pressure changes along the capillary had been the controlling influence. In this respect the findings were identical with those in the skin. The possibility is far from being excluded that the spread of dyes from frog blood results in some part from a filtration of colored water under pressure; but such filtration, if it occurs, may be most abundant where the intracapillary pressure is lowest, that is to say at the further end of the capillary.

The important rôle of diffusion in vital staining has been acknowledged since Schulemann's basic contribution on the theme (17). Our findings fall in with his generalization that in the absence of specific

EXPLANATION OF PLATES

PLATE 11

FIG. 1. Superficial capillary meshwork in the skin of the abdomen of *Rana pipiens*, after injection of an ink-gelatin mass. Preparation mounted without clearing or staining. It has precisely the appearance noted prior to the escape of a deep-colored dye from the blood of the living animal. Where the lines of the arrows meet are some converging venules incorporated in the network. Other efferent and afferent vessels connect with it from beneath and hence are not visible. The capillary meshes which encircle glands are rounded and slightly smaller than the others. $\times 22$.

FIG. 2. Superficial capillary network in a transparent region of the thigh skin of *Rana clamitans*. An India ink mass was followed by one of gelatin-carmines to differentiate venules and arterioles. The capillaries containing red mass are far less plainly visible than those holding black. (Fig. 9 shows the network in detail.) Everywhere foramina pierce the stratum compactum. A group of contracted pigment cells obscures the vessels near the middle of the left edge of the preparation. The bright spots here and there are due to "interference cells." Specimen photographed while fresh. $\times 14$.

FIG. 3. Cleared skin of the thigh of *Rana clamitans* viewed from beneath to show the distribution of vessels in the tela subcutanea. The small vessels ramifying against the dimly seen, superficial rete of the corium appear to end abruptly because they pierce the stratum compactum at right angles to supply this rete. $\times 22$.

FIG. 4. Distribution of trypan red from a rete like that in Fig. 1. Curarized *Rana pipiens* with brain pithed, injected in the course of 38 seconds; photograph taken $4\frac{1}{2}$ minutes later from the living animal. Already much dye has escaped from the blood into the tissue supplied from the further portion of the rete, and the plasma has become so far decolorized that the proximal capillary meshes are no longer plainly visible. The tissue supplied by them contains no stain. In the stained regions the capillaries and venules appear broadened and blurred by perivascular dye. See also Fig. 7. $\times 14$.

PLATE 12

FIG. 5. Distribution of Congo red from the rete on the belly of a curarized *Rana pipiens*. The injection required $\frac{3}{4}$ minute and the picture was not taken until $9\frac{1}{2}$ minutes later. Escape of dye into the tissues has just begun, from the further portion only of the capillary network and from gland circlets here and there. The arrows point to such circlets. The blood still contains so much Congo red that the network stands out as if injected, the meshes nearer the veins appearing relatively broad because of spread of the dye. $\times 14$.

FIG. 6. Further stage in the staining of the same frog; photograph taken from the living animal 12 minutes after Fig. 5. The color pattern has now been rendered irregular by the escape of dye from gland circlets. Everywhere else the

SUMMARY

A steeply mounting gradient of permeability is demonstrable along the meshwork of capillaries which connects the arterioles and venules of the skin of the frog. The venules incorporated in the meshwork are even more permeable than the capillary meshes giving into them.

The presence of the gradient under such differing conditions as exist along frog and mammalian capillaries enables one to rule out certain factors which might be invoked to explain it; and it is not explainable in terms of those influences generally recognized as conditioning exchange between the blood and tissues. Not improbably it results from a structural differentiation along the capillary.

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PLATE 14

FIG. 13. Skin of the belly of *Rana pipiens* after injection of the vessels with an India ink mass. The capillary rete lies immediately under the epidermis, with numerous "interference cells" at the same level, having gray granules. A large vessel is to be seen in the tela subcutanea. $\times 170$.

FIG. 14. Injected skin of the thigh of *Rana clamans*, highly magnified, to show a venule piercing the stratum compactum and the cross-section of a capillary in the rete. The latter—recognizable by the black plug of India ink—is separated from the venule by the cross-section of a gland. Hematoxylin and eosin. $\times 360$.

FIG. 15. Another part of the same preparation to show an emergent arteriole and a neighboring arteriolar capillary. Near the middle of the picture is a gland in cross-section and to its right another capillary. $\times 360$.

Congo red has got out only from the further portion of the rete, the tissue about its proximal region being unstained. The rete here is lighter than in Fig. 5 because of a partial decolorization of the blood. $\times 14$.

Fig. 7. Trypan red staining, in the gross. Photograph of the frog furnishing Fig. 4, taken from the living animal 10 minutes later. The distribution of the dye was essentially unchanged. The skin surface was peppered with red dots on a pale ground, numerous over the abdomen, where was some general staining also, and scattered over thorax, thigh, and throat. In the lower leg the circulation was poor and little staining had occurred. On the inner side of the thighs, on the other hand, it was confluent and so intense that the skin appears black in the photograph. Natural size.

Fig. 8. Distribution of trypan red to the skin of the upper thorax of *Rana pipiens*,—for comparison with Figs. 4 and 7; photograph taken 10 minutes after the dye injection. Less of the dye had got out than on the abdomen of the same animal, and into a smaller region. The capillaries appear broader in the stained skin than elsewhere, owing to stain in their walls. $\times 14$.

PLATE 13

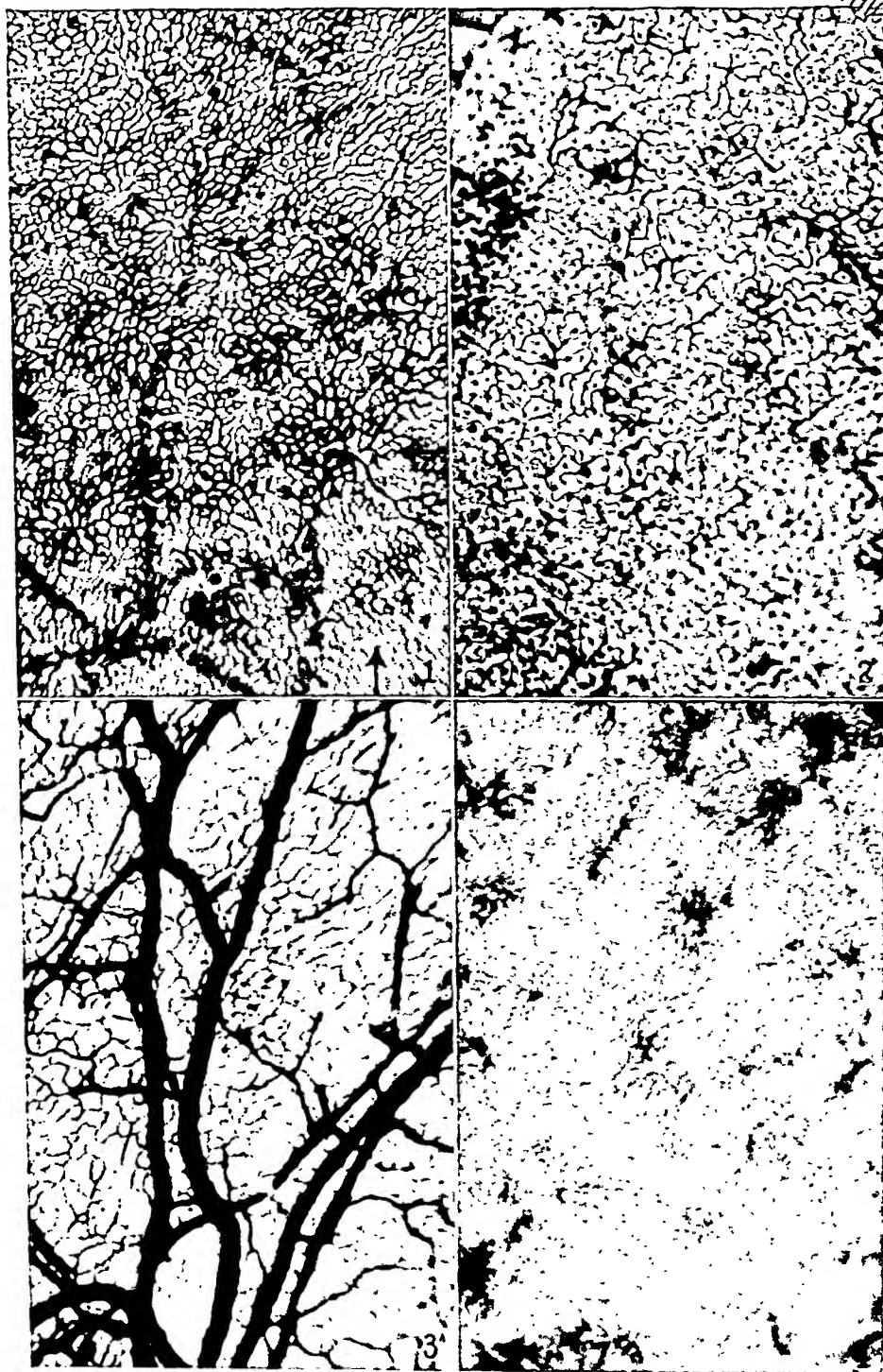
Fig. 9. A higher magnification of the skin of the preparation shown in Fig. 2. The big arrow points to an especially large opening in the "sieve layer" of the stratum compactum from which an artery (not seen) emerges to join a radiating capillary meshwork recognizable by the light hue of the red mass it contains. The capillaries can be traced in several directions to venules, distinguishable by their black content, which disappear into holes in the compactum. The small arrows point to emergent arterioles. $\times 46$.

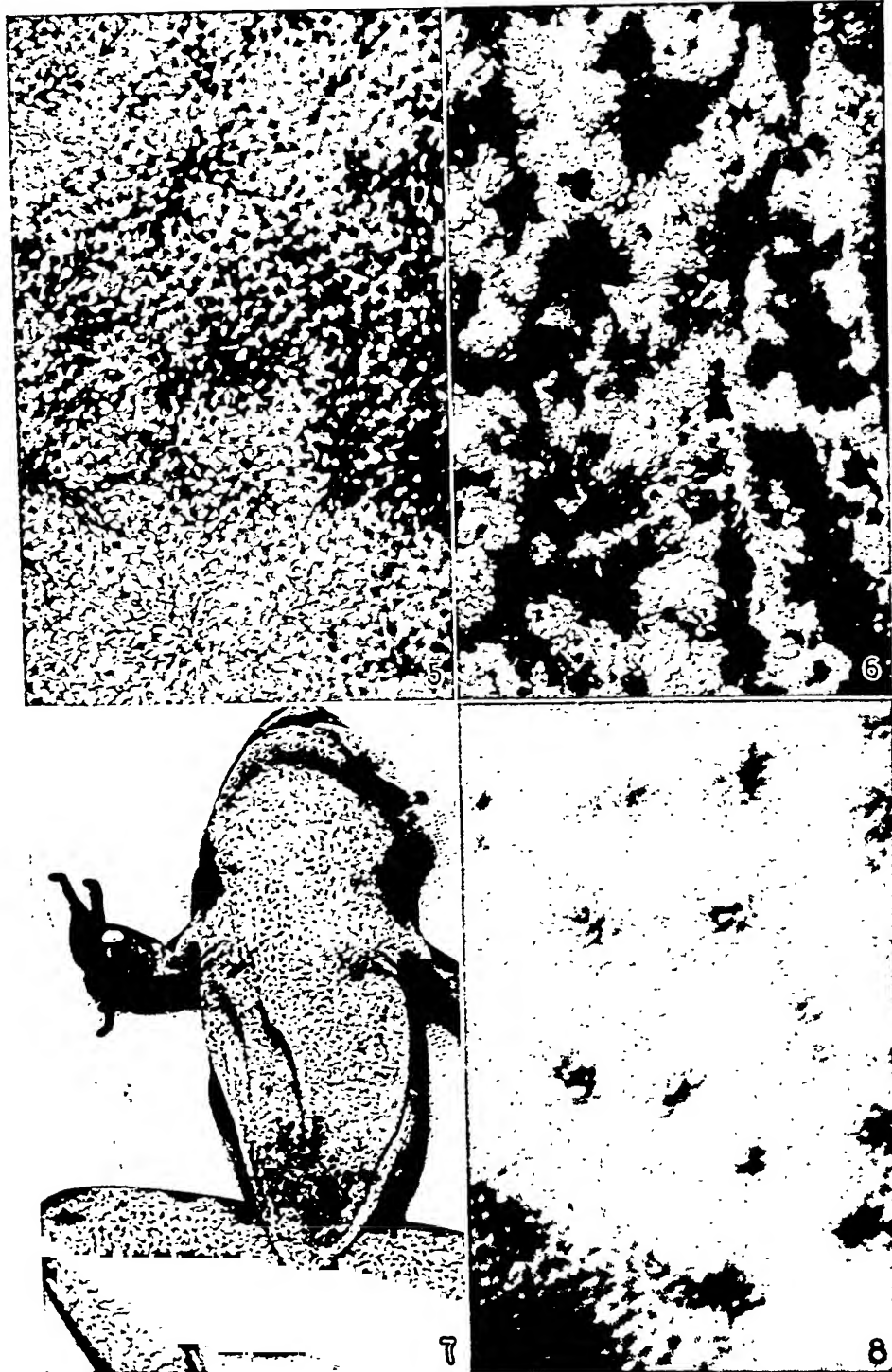
Fig. 10. Cross-section of the skin of the thigh of *Rana clamitans* in a region like that of Fig. 9; vessels injected with an India ink mass. The capillaries of the rete lie immediately beneath the epidermis, and are recognizable by the plugs of black in their lumen. Two vessels pierce the stratum compactum to reach the rete; like it they are walled by a single layer of cells. Only one of the glands lying in the loose spongiosum is cut through its lumen. The thin tela subcutanea and the dense, superficial "sieve layer" (*Siebschicht*) of the stratum compactum should be noted. Hematoxylin and eosin. $\times 192$.

Fig. 11. Skin of the belly of *Rana pipiens*. Uninjected specimen showing the same layers as in *clamitans*. Three glands are shown cut across. The tela subcutanea is relatively thick. The capillaries cannot be discerned, but an artery pierces the stratum compactum. Hematoxylin and eosin. $\times 170$.

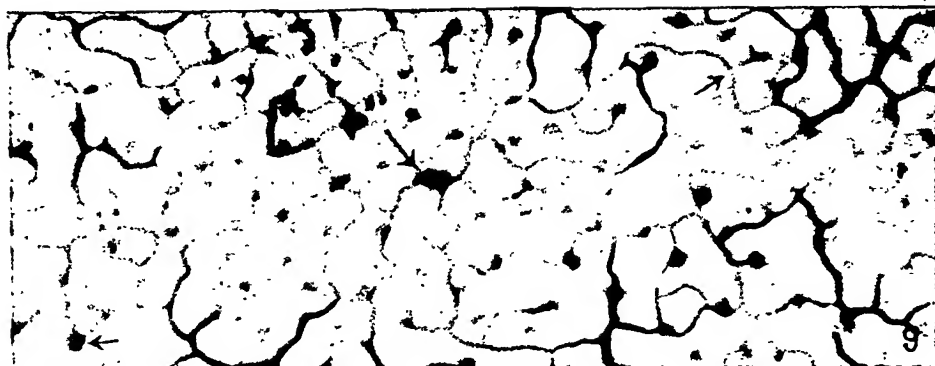
Fig. 12. Skin of the belly of *Rana pipiens*. Photographed to bring out in black the layers of "interference cells," just beneath the epidermis and in the tela subcutanea. The tissue is swollen as result of treatment with chemicals. Picric acid stain. $\times 170$.

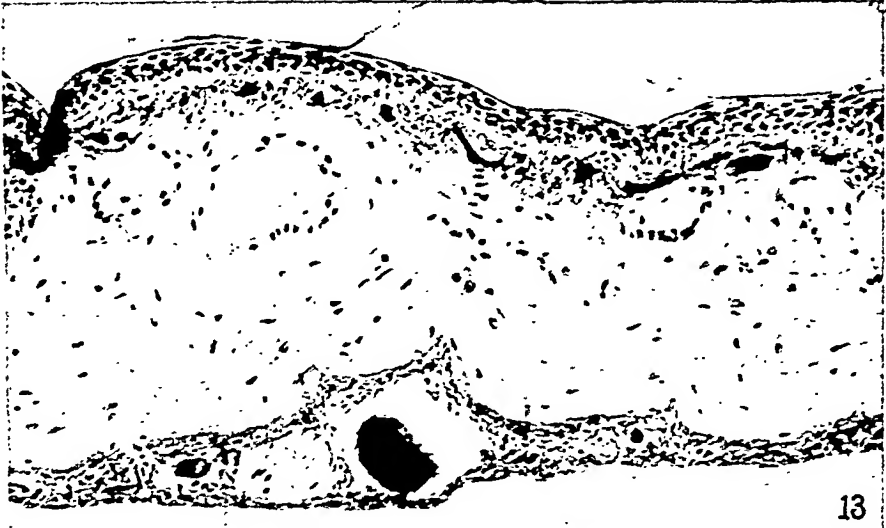












of experimental leucemia (18) pointing to unsolved problems accessible to experimentation. Other phases of leucemia research were at the same time reviewed by Dr. Whitney (28) and Dr. Farley (7). It has been my privilege to undertake some of the problems outlined in Dr. Opie's work.

HISTORY

The literature on the leucemias of fowls has been extensively reviewed by Ellermann (5) and Schmeisser (21) and more recently by Tio Tjwan Gie (26) as well as by Opie (18). In the following summary reference will be made to the more recent communications and to the more important contributions of the older literature.

Moore (17) described as "infectious leucemia" a disease now called fowl typhoid fever, which is accompanied by a rise of the polymorphonuclear leucocytes in the circulating blood to more than 200,000 white blood cells per cubic millimeter. The possibility of causing leucemia by small amounts of *B. sanguinarium* was re-investigated by Winternitz and Schmeisser (29). There is no evidence to show that *B. sanguinarium* or related microorganisms are capable of bringing about a proliferation of immature white cells.

Butterfield (4) gave the first accurate description of aleucemic lymphadenoma of the fowl and Warthin (27) was the first to describe a case of leucemia in fowls, which, he thought, was of the lymphoid type.

The most valuable contributions to the subject are those of Ellermann and Bang (5, 6), whose main observations may be summarized as follows. Leucosis* is transmissible from fowl to fowl by injections of blood, organ suspension, or cell-free Berkefeld filtrate of these materials. It is therefore due to a filterable virus. The percentage of successful inoculations with blood or organ suspension is from 22 per cent to 41 per cent, and of inoculations with Berkefeld filtrate, about 17 per cent. The birds that do not develop leucemia after one inoculation are susceptible to reinjections. The duration of illness is from 4½ to 5 months in the first passage with a decrease on subsequent passages to about 1 month, when it becomes fixed. The same virus, they thought, may give rise to three types of leucosis, lymphoid, myeloid, and erythroleucosis. The first two types are similar to the same diseases of man. In lymphoid leucosis, in Ellermann's experience,

* The collective term leucosis is used as proposed by Ellermann for all types of leucemia, lymphomata, and allied conditions. Lymphoid leucosis includes leucemic and aleucemic tumor-like proliferative processes involving the lymphoid system; myeloid leucosis, those that involve myelocytes and their precursors. The term leucemia is used only when there is a progressive increase in the blood of cells containing no hemoglobin. The adjective lymphoid is used to designate all cells that resemble medium or large lymphocytes; some of these, however, which could be identified as the earliest forms of erythroblasts, will be spoken of as lymphoid erythroblasts.

BOSTON PSYCHOPATHIC HOSPITAL

OBSERVATIONS WITH A NEW TRANSMISSIBLE STRAIN OF THE LEUCOSIS (LEUCEMIA) OF FOWLS*

By J. FURTH, M.D.

WITH THE ASSISTANCE OF CHARLES BREEDIS AND RUTH KLINGELHOFFER

(From The Henry Phipps Institute, University of Pennsylvania, Philadelphia)

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Since the report of Ellermann and Bang (1908) (6) on the transmissibility of the leucemia of fowls only Schmeisser (21) has been able to transmit one spontaneous case. The strains obtained by these investigators have long ago died out and those who have attempted to recover a new transmissible strain report failures. Moreover the observations of Ellermann could only in part be confirmed by his collaborator (1) (see page 257). Thus doubts have arisen that leucemia of the fowl is transmissible (14, 15). Attention has been called to the occasional high incidence of spontaneous leucemia and to the fact that, with one very recent exception (1), none of those attempting transmission suggested the possibility of the development of leucemia in their laboratory animals through causes other than inoculation.

Investigation of the leucemia of fowls is beset with many difficulties, such as to obtain a uniform stock of chickens throughout the year, and to keep them in good health under laboratory conditions, especially to prevent epidemics, such as infectious tracheitis, which twice with an interval of 1 year destroyed most of our birds. These obstacles may serve to explain in part why many of the problems implicit in the experiments reported here cannot yet be answered with certainty.

The present paper describes a new transmissible strain of the leucemia of fowls. The various types of leucemia observed will be described in subsequent reports under the headings *erythroleucosis*, *myeloid leucosis*, and *lymphoid leucosis*.

A special fund donated for the study of the leucemias and related conditions enables us to take up research along these lines. Dr. Opie has surveyed the field

* This investigation has been supported by a Fund for the Study of Leucemia and Related Diseases.

undertaken with blood drawn from live birds or with material from organs removed immediately after death.

Bleeding of the Fowl.—With the following technic 20 to 40 cc. blood can be easily removed from live birds without any serious damage to them. Syringe and needles (gauge 20 or preferably 19) are chilled and the desired amount of blood is taken up into the syringe in one-tenth its volume of heparin solution (0.1 per cent). The needle is inserted into the brachial vein near the shoulder joint in a direction opposite to the blood flow, so that the blood can be diverted into the needle by a slight pressure on the vein.

The birds were injected soon after they were purchased. With the exception of eight White Leghorn fowls, of which four were used in the first passage, and four in passage III-B, all of them were Barred Plymouth Rocks, weighing at the time of injection from 700 to 1000 gm. The majority were purchased from one farm in Delaware.

Blood examinations were made of all birds that appeared to be ill. Blood smears were taken from all birds before inoculation and occasionally from the entire flock. The blood films were stained either with Wright's solution alone or in combination with Giemsa's solution as follows—with Wright's solution for 3 minutes and after the addition of an equal amount of distilled water stained for 3 more minutes, then washed in water and stained for 5 minutes with Giemsa's solution (1 drop to 1 cc. of distilled water).

Counts of white and red corpuscles were made by diluting the blood 1:100 with Toisson's solution.

Transmission of Leucosis of Fowls

Inasmuch as the character of erythroleucosis and myeloid leucosis will be described more fully in subsequent publications, the data presented here are intended chiefly to demonstrate their transmissibility, the relative frequency of transmission after inoculation of cellular and other material, and briefly to show the effect of various treatment on the transmissible agent and its filterability. A brief description of different types of leucosis as they have appeared in these experiments follows.

Myeloid leucemia appears to begin with a tumor-like proliferation of the myelocytic and myeloblastic elements of the bone marrow. The marrow may show as much hyperplasia as is seen in advanced cases, yet the blood picture may appear to be normal and myeloid foci in internal organs may be wanting. This state may be designated "incipient myeloid leucemia" and is found in inoculated birds that die of an intercurrent disease a short time after inoculation. Those cases in which myeloid hyperplasia was advanced but not extreme are classified as negative but mention is made of the possibility that inoculation has been successful.

the circulating blood is never involved. Erythroleucosis is a condition peculiar to avian pathology, in which primitive lymphoid cells, considered by Ellermann hemoglobin-free precursors of erythrocytes, crowd the capillaries of various organs. Erythroleucosis is always accompanied by a severe anemia. Pure anemia, without proliferation of lymphoid erythroblasts, likewise occurs after inoculation with the virus of fowl leucosis and is considered by Ellermann as an extreme type of erythroleucosis.

The transmissibility of avian leucemia has been confirmed by Hirschfeld and Jacoby (12) and Burckhardt (3), whose strains originated from Ellermann's. Schmeisser (21) succeeded in transmitting a spontaneous case of myeloid leucemia. The disease produced by the injections of organ emulsions resembled in all respects the spontaneous case. Neither Hirschfeld and Jacoby nor Schmeisser* succeeded in transmitting fowl leucemia by Berkefeld filtrates.

Ellermann's classification of the leucemias was given little consideration by subsequent workers, who apparently failed to recognize cases of erythroleucosis and grouped them either as myeloid or as lymphoid leucemia. Pickens (20), for example, considered all the 22 spontaneous cases observed by him as lymphoid. McGowan (16), on the other hand, thought that the 150 instances of spontaneous avian leucemia examined by him belonged either to the myeloid or mononuclear, but not to the lymphoid type, and he expresses the view that leucemia of fowls is a single pathological complex but has a multiple etiology.

Pappenheimer (19) observed the frequent association of fowl paralysis with lymphomatous infiltration of various organs. His experiments suggest that a filterable virus is the causative agent, but that it does not produce leucemia. Mathews and Walkey (15) have failed to reproduce lymphadenoma by inoculating 22 birds in three experiments. In opposition to Ellermann, they assume that lymphadenoma of fowl is a neoplasm not caused by an infectious agent. Mathews (14) tried, without success, to transmit "leucochloroma" of the fowl from three spontaneous cases to 23 birds. The leucochloroma of Mathews is evidently the myeloid leucosis of Ellermann.

Andersen and Bang (1), modifying Ellermann's classification, differentiate between intravascular and extravascular leucosis, the former comprising both myeloid and erythroleucosis. They report negative results in an attempt to produce leucemia by way of the alimentary tract in 22 fowls. Transmission by mites was also unsuccessful.

EXPERIMENTAL

Material inoculated.—In view of our failure, prior to the recovery of the transmissible strain reported here, to transmit leucemia by inoculation of postmortem material, the transmissions in these series were

* Personal communication.

roleucosis but the hyperplasia of the marrow is to a considerable extent also myelocytic and myeloblastic. These as well as most of the borderline cases were grouped as mixed forms.

Myeloma.—In a few instances, scattered in various parts of the body, most consistently in the blood-forming organs, tumor nodes or nodules were found that were composed of myeloblasts and myelocytes and were accompanied by only slight involvement of the blood. If the tumors were composed entirely of myelo-

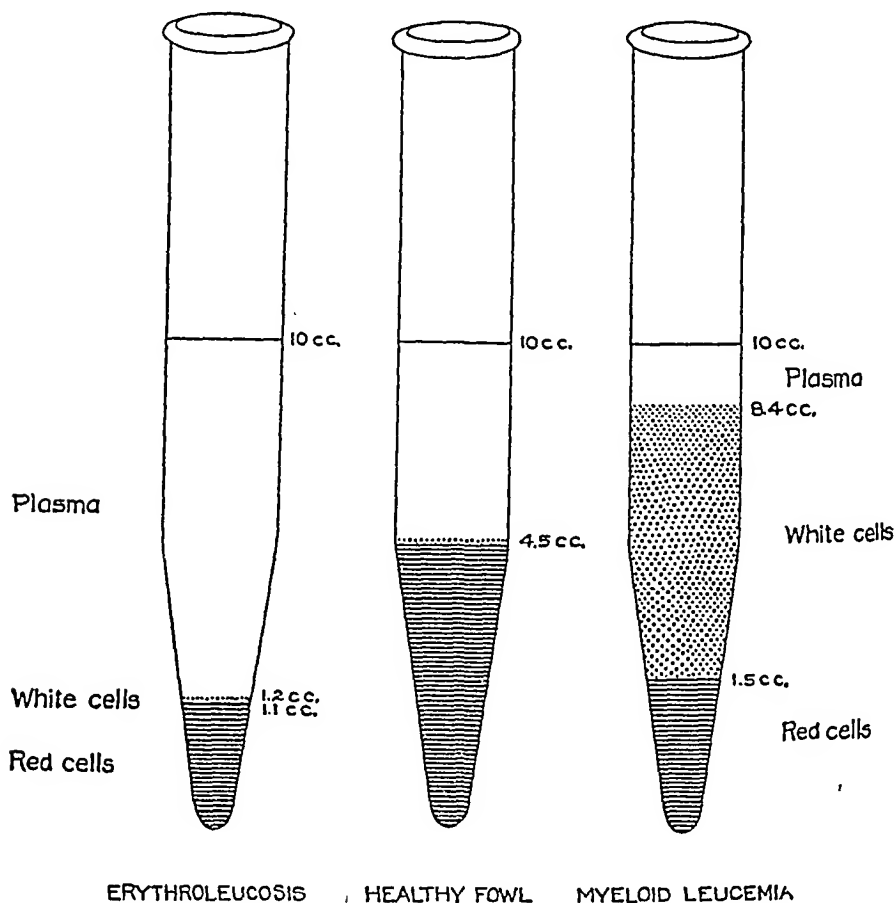


FIG. 1. Showing the red cell, white cell and plasma layers of the centrifugalized blood in healthy fowl, in erythroleucosis, and in myeloid leucemia.

cytes they will be referred to as myelocytoma. The relation of these tumors to the transmissible leucemia of the fowl is under discussion. (See page 264.)

Lymphoid leucosis has been defined with Ellermann as the condition in which there is a diffuse or nodular, tumor-like invasion of various organs with large lymphocytes. The liver has been the organ most frequently and extensively involved. In no case observed by us was a leucemic involvement of the blood established with

The blood invasion was found to constitute the next stage and a terminal stage is characterized by heterotopic myeloid foci, most frequently in the liver. Most of the birds with myeloid leucemia die in this stage. The character of the cells, the leucemic blood picture, and the characteristic bone marrow hyperplasia leave in most cases no doubt as to the nature of the condition. Blood counts are usually high, the ratio of white cells to red cells reaches frequently 1:3, and in some instances the number of white cells exceeds that of the red cells.

Erythroleucosis could usually be diagnosed from blood films by the presence of erythroblasts in various stages of development, proerythroblasts, and primitive lymphoid cells. These cells are defined and illustrated in the report on erythroleucosis (9, b). In some instances, the primitive cells are in such number that the blood picture appears leucemic. In secondary anemias the number of erythroblasts is usually high but polychrome erythroblasts are few and basophilic erythroblasts occur rarely if at all, and cells with the character of proerythroblasts are practically absent. These differences in the blood smear permit the differentiation of erythroleucosis from these conditions in most if not in all instances. In sections, erythroleucosis is characterized by a selective accumulation of primitive lymphoid cells (Ellermann's lymphoidocytes*) in the capillaries of various organs, chiefly the bone marrow, liver, and spleen. These are assumed to be hemoglobin-free precursors of erythrocytes. The purely intravascular nature of the process as well as the character of the cells make the diagnosis in the majority of instances unmistakable. I have been unable to reproduce the microscopic characters of erythroleucosis by repeated bleeding or prolonged administrations of blood poisons, such as pyrocin.

Nothing demonstrates more clearly the essential difference between erythroleucosis and myeloid leucosis as produced by transmission than spinning of the blood. On inspection of the centrifugalized tubes (see Fig. 1) it becomes obvious that the former process is anemia and the latter, leucemia.

Mixed Cases of Myeloid and Erythroleucosis.—Erythroleucosis and myeloid leucosis appear to be intimately related. It is not uncommon to find a few myelocytes in the blood of birds with erythroleucosis and occasional erythroblasts are frequently found in myeloid leucemias (see 5, b). In some cases the microscopic picture of the blood and also of the organs is a combination of the two processes. Since there is a continuous series between erythro- and myeloid leucosis their separation into pure and mixed forms is more or less arbitrary. The cases that were grouped here as mixed forms show an extreme hyperplasia of the bone marrow formed by lymphoid cells in sinusoids as well as myelocytes and their precursors in the trabeculae; the leucemic blood appears to be a mixture of erythroblasts in various stages of development, primitive large mononuclear cells (myeloblasts), and myelocytes. Extravascular myeloid foci are found in the liver. Particularly difficult is the classification of those cases in which the blood smear is that of eryth-

* McGowan and Andersen and Bang identify these cells with hemocytoblasts.

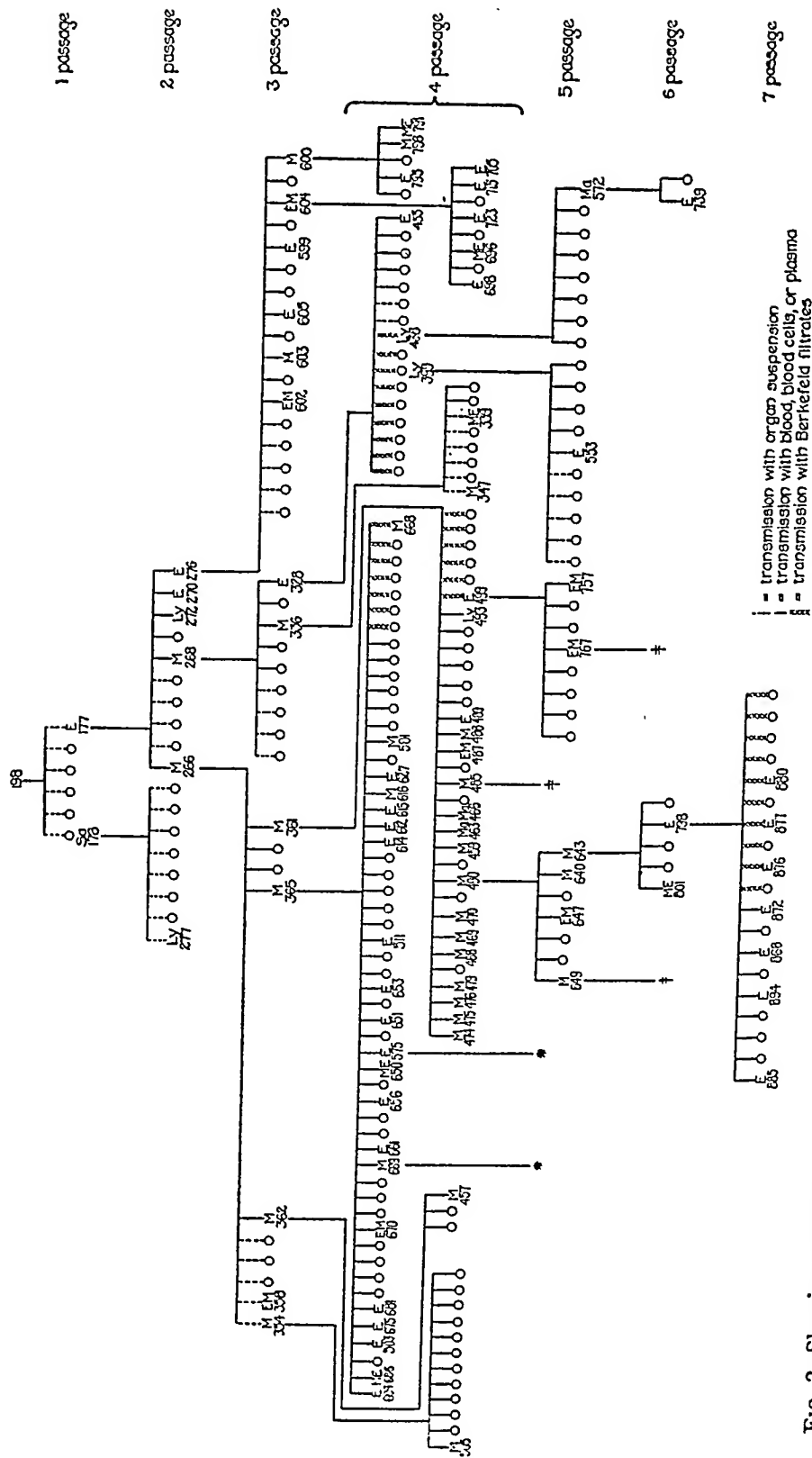


FIG. 2. Showing passages of a transmissible strain of leucosis of fowls. † = continued at the Henry Phipps Institute, * = continued at the Veterinary School of the University of Pennsylvania, circles indicate unsuccessful transmissions, M = myeloid leucosis, ME = combination of myeloid and erythroleucosis, E = erythroleucosis, Ly = lymphoid leucosis, Sa = sarcoma, Ma = myeloma, E = erythroleucosis, Ly = lymphoid leucosis.

certainty. Evidence will be presented suggesting that lymphoid leucosis is not caused by the transmissible agent of the leucemia of fowls.

Of 105 cases of leucosis occurring in the 30 transmissions described here, 34 have been classified as myeloid leucosis, 36 as erythroleucosis, 25 as mixed forms, 4 as myeloma, and 6 as lymphoid leucosis.

The Origin of the Transmissible Strain

The bird to which the transmissible strain may be traceable was a White Leghorn hen (198) examined in the laboratory after death. The liver and spleen showed no gross abnormalities. The femur was thickened and its cavity was almost entirely replaced by bone so that no free marrow could be obtained. The kidneys were greyish, soft, and enlarged, particularly the upper lobes. The heart was greyish, slightly enlarged, and showed a few slightly prominent greyish nodules. In the other organs, including the liver and spleen, no gross abnormalities were noticed. Microscopic examination revealed an extensive diffuse infiltration with lymphoid cells of the kidney, heart, thyroid, and parathyroid glands, a similar nodular and perivascular infiltration of the liver and some infiltration of lumbar nerves and ganglia adjacent to the adrenals. The number of white blood cells in the blood stream, as seen in sections, was not increased.

Emulsions of the kidneys, which showed the most extensive lymphoid infiltration, and of the liver were injected into four White Leghorn and three Barred Plymouth Rock chickens. One of the latter (177) developed a severe anemia in about $2\frac{1}{2}$ months. The red blood count was 445,000; the white blood count 57,000, and the figure for hemoglobin (Sahli) 15. There were many polychrome red cells, and polychrome and basophilic erythroblasts in the circulating blood. On microscopic examination, the capillaries of the blood-forming organs, kidneys, and to a lesser degree those of the lungs, heart, and thymus, were distended with lymphoid cells and the lesions had the characters described by Ellermann as intravascular lymphoid leucosis, which he regards as a severe form of erythroleucosis.

In view of the fact that only one of the inoculated birds developed leucosis and that the transmission was made 10 to 20 hours after death it remains uncertain whether the changes in this chicken (177) were in reality the result of passage or a spontaneous disease. All subsequent cases are derived from 177.

Another case (277) of probably spontaneous lymphoid leucosis, shown in Table I, developed in a fowl that was inoculated with a sarcoma of the Rous type occurring in the experimental strain. In the series of Dr. Stubbs (24) the incidence of lymphoid leucosis among the inoculated fowls was the same as among the controls. Among the 193 birds, then, two cases occurred of that type of leucosis that may be assumed to be spontaneous, and not one spontaneous case of the two types that have been shown to be transmissible.

TABLE I
Incidence of Leucosis in Two Control Series of Fowls

	Observed in the laboratory					Incidence of leucosis
	$\frac{1}{4}$ to 1 mo.	1 to 3 mos.	3 to 6 mos.	Over 6 mos.	Total	
A. Number of uninoculated chickens.....	27	28	16	8	79	No leucosis
B. Chickens inoculated during miscellaneous transmission experiments.....	22	13	32	47	114	One lymphoma
Total.....	49	41	48	55	193	

Incubation period of transmissible leucosis

	$\frac{1}{4}$ to 1 mo.	1 to 3 mos.	3 to 6 mos.			
	per cent	per cent	per cent			
	41	49	10			

Erythroleucosis and myeloid leucosis are caused by the same transmissible agent. The data presented above and given in the subsequent paper (9,b) demonstrate the transmissibility of the leucosis of fowls. One remarkable feature of the agent responsible for the transmission is that it affects the two systems involved in the formation of erythrocytes and of granulocytes. The reports of Ellermann and of Schmeisser and the evidence presented in Tables II, *a* and II, *b* leave no doubt as to the correctness of this statement.

The factors determining whether the one or the other of these systems or both of them become involved are at present entirely undeter-

With the blood of 177, suffering with erythroleucosis, six birds were inoculated, each being given from 2 to 4 cc. of the unclotted blood, and of these two developed myeloid leucemia and two erythroleucosis. This result and the subsequent transmissions reported here furnish sufficient evidence that myeloid and erythroleucosis are transmissible. It must, however, be kept in mind that a few cases of leucosis in these series of transmissions might have developed spontaneously.

Fig. 2 is a survey of the 30 transmission experiments of the first seven passages; it gives the designation of those birds from which further passages have been made and shows their relation to Birds 198 and 177, from which the transmissible strain was recovered.

The salient data of the individual passages will be presented in Tables II, *a* and II, *b*. The fowls that died of an intercurrent condition within 30 days after inoculation have been omitted from Fig. 2 if not showing leucotic changes.

Control Series

The results of the transmissions cannot be interpreted without a knowledge of the incidence and type of leucosis occurring spontaneously under the conditions given.

Arrangements made to determine accurately the occurrence of spontaneous leucosis among chickens when kept in the laboratory were sacrificed during a severe epidemic of infectious tracheitis, with the purpose of saving the transmissible strain. This study was later undertaken with Dr. Stubbs and will be reported in a subsequent paper (24). A fairly large number of birds, however, may be presented here as control material. They will be collected into two groups, A, uninjected controls, B, fowls used in experiments undertaken in the attempt to transmit spontaneous, mainly lymphomatous tumors.

In Table I, the control birds are presented, grouped according to the length of time they were observed in the laboratory.

Discussion of the Transmission Experiments

It is noteworthy that neither erythroleucosis nor myeloid leucosis was observed among 79 uninoculated fowls and among 114 fowls receiving material other than that derived from the transmissible strain. In the latter series of "controls" three tumors were found but only one (102) of these, lymphosarcoma, falls within the term leucosis.

TABLE II, a
Incidence of Leucosis among the Passages of the Transmissible Strain

Passage	Donor	Blood of donor at time of transfer	Material inoculated	Route of inoculation	No. of birds inoculated	No. lost by inter-current death	Results					
							Negative	Myeloid	Erythro	Myelo and Erythro	Myeloma	Lymphoid
I	198	Appears normal	Organ emulsion "	i. v.	6	1	4*		1	1		
				i. p.	1							
II-A	177E	W. B. C. 57,000	Whole blood	i. v.	8	2	1	2	2			1
		R. B. C. 445,000 Hb. 15			4		4					
II-B	176Sa	Appears normal	Organ emulsion	i. v.								
					6		5					1
III-A	268M	W. B. C. 430,000 R. B. C. 1,760,000	Tumor	i. musc.								
					6	2†	3	1				
IV-A	336M	W. B. C. 328,000 R. B. C. 1,290,000 Hb. 25	Whole blood	i. v.	6	1	4†		1			
				i. v.	6							
IV-C	362M	W. B. C. 550,000 R. B. C. 645,000 Hb. 36	Organ emulsion	i. v.	3	1	2					
					7	1	4	2				
IV-D	361M	W. B. C. 300,000 R. B. C. 1,520,000 Hb. 39	Blood cells	i. p. or i. v.	2		1	1				
					1		1					
			Plasma									
					7	3		4				
			White cell layer		6	2	1	3				
					6		3	1			2	
			Red "									
					6							
			Plasma									
					6							

mined. It is evident from Table I that the type of leucosis of the donor is not the determinant.

Lymphoid leucosis is apparently not caused by the virus of the leucosis of fowls. In the experimental series six cases of lymphoid leucosis have been observed among 377 fowls receiving material from myeloid or erythroleucosis and two cases of lymphoid leucosis were found among 193 control fowls. Similar figures are presented in the subsequent series (24) of experiments, in which two cases of lymphoid leucosis were found, one among 25 fowls inoculated with the transmissible agent of the leucosis of fowls and one among an equal number of uninjected controls.

Lymphoid leucosis appears to constitute the most common neoplastic disease of the fowl. Tyzzer and Ordway (25) found seven among nine, Feldman (8) 11 among 19 tumors composed of cells like lymphocytes. Mathews and Walkey (15) observed that in certain flocks lymphadenoma may cause a mortality of from 5 to 30 per cent during a 60-day period, or over a period of 6 months. Kerr (13) finds the incidence of death is 9.8 per cent and Schneider (22) 2 to 3 per cent. Hare (10), working in cooperation with us, found that 4 per cent of the total death of fowls in Delaware is due to leucemias and lymphomata, about half of these being lymphoid leucosis.

Ellermann, who maintained that all types of leucosis are caused by one virus, presents no data concerning the incidence of spontaneous neoplasms among the birds he used in his experimental studies, but the number of cases of lymphoid leucosis occurring among his inoculated fowls is not beyond the expectancy of their spontaneous development. Hennepe's observations (11) made in a neighboring country, Holland, showed that 7 per cent of all deaths were caused by leucemia and 1.8 per cent by tumors. In comparing these figures with the data given above, it should be taken into consideration that about 75 per cent of our inoculated fowls were killed while healthy to terminate experiments and many others suffered unnatural deaths unavoidable with experimentation.

It appears that lymphoid leucosis is as common in the control series as it is among the birds inoculated with material from myeloid or erythroleucosis. Nevertheless, transmission experiments with the two cases of lymphoid leucosis given in Table I (399, 438) seem at first sight to contradict the opinion that lymphoid leucosis is not caused

TABLE II, *a*—*Concluded*

Passage	Donor	Blood of donor at time of transfer	Material inoculated	Route of inoculation	No. of birds inoculated	No. lost by inter-current death	Results					
							Negative	Myeloid	Erythro	Myelo and Erythro	Myeloma	Lymphoid
V-C	505M	W. B. C. 161,000	Bone marrow	i. v.	5		4			1		
V-D	438L	Leucocytosis	Organ suspension	i. v.	9		8				1	
V-E	490M	W. B. C. 92,000	Blood cells	i. v.	3		1	2				
		R. B. C. 1,090,000 Hb. 25	"Hemolyzed" blood cells	i. v.	4		2	1		1		
V-F	499EM	W. B. C. 214,000	Whole blood	i. v.	4	1	2					
			Blood cells	i. v.	3	1	1		1			
		R. B. C. 1,128,000	Plasma	i. v.	4	1	2			1		
V-J	466EM		Bone marrow	i. marrow	2		2					
VI-A	572MaE	W. B. C. 131,000 R. B. C. 1,535,000	Whole blood	i. v.	6	4	1			1		
VI-B	643M	W. B. C. 650,000 R. B. C. 1,150,000	" "	i. v.	5		2	2	1			

IV-G	365M	W. B. C. 52,000 R. B. C. 1,600,000 Hb. 38	Blood cells	i. v.	5		4†	1
IV-II	365M	W. B. C. 214,000	Plasma	i. v.	3		2	1
		R. B. C. 1,240,000 Hb. 28	Blood cells	i. v.	5		1†	1
IV-K	365M	W. B. C. 369,000 R. B. C. 369,000	Frozen blood cells	i. v.	5		3	2
IV-L	365M	About as above	Whole blood	i. v.	9	4	2	1
IV-M	604EM	W. B. C. 203,000	Whole blood	i. v.	4	3		1
		R. B. C. 316,000 Hb. 65	Blood cells	i. v.	6	2	2	1
IV-N	604EM	W. B. C. 940,000	Plasma	i. v.	4	3		1
		R. B. C. 565,000	Blood cells	i. v.	5	3	1	1
IV-O	600EM	W. B. C. 455,000	Plasma	i. v.	5	3		1
		R. B. C. 995,000 Hb. 20	Whole blood	i. v.	5		2	1
V-A	459M	W. B. C. 106,000 R. B. C. 2,470,000	Blood cells	i. v.	4	3	1	
V-B	399L	W. B. C. 62,500	Frozen blood cells	i. v.	4	2	2	
		R. B. C. 2,135,000 Hb. 39	Whole blood	i. v.	4		4	
			Organ emulsion	i. v.	6	5		1**

The abbreviations are identical with those of Table I. * One died of Sarcoma (Rous type). † One of these might be incipient M or E. ** With sarcoma of the ovary.

by the transmissible agent described, for each of these lesions when inoculated, one into 9, and one into 10 normal fowls, gave rise to one case of leucosis. One was erythroleucosis (553) ending in complete recovery; the other (572) was myeloma. Connection with the transmissible agent of leucosis of fowls is uncertain.

Of particular interest in this connection is the recently reported work of Andersen and Bang (1). They have tried to transmit 21 spontaneous cases of leucosis, of which 10 were aleucemic (lymphoid) and 11 myeloid or erythroleucosis, but of these only five of the leucemic cases proved to be transmissible. They emphasize their inability to transmit "leucoblastic" (lymphoid) leucosis and conclude that the chronic type of leucosis,* although caused by a filterable virus, is not transmissible. Their observation may better be interpreted as supporting the view that aleucemic lymphoid leucosis is not caused by the transmissible agent of the leucosis of fowls.

Relation of the Agent Transmitting Leucosis to Organs, Blood Cells, and Plasma

In order to determine the incidence of successful transmissions with reference to the material inoculated the passages have been analyzed in two tables. One of these (Table II, *b*) includes the eight transmissions made from five cases of myeloid and three of erythroleucosis, which include Berkefeld filtrations. In Table II, *a* the transmissions made from the spontaneous case, two cases of erythroleucosis, 14 cases of myeloid leucosis, two of mixed myeloid and erythroleucosis, two of lymphoid leucosis, one myeloma and one sarcoma are collected. The results are then summarized in Table III.

It is seen from Table III that when whole blood was used for inoculation, about 50 per cent of the birds that did not die from an intercurrent disease within 30 days after inoculation developed either myeloid or erythroleucosis or a combination of these two conditions. The result of the inoculation was the same if not better when instead of whole blood, blood cells were injected.

The serum was removed by centrifugalization for about 5 minutes at about 1000 R.P.M. and the cells were taken up in the original volume of normal serum or Locke solution. In Experiments IV-N and IV-G, the cells have been subjected to an additional washing with Locke solution.

* I do not find evidence to substantiate the view that lymphoid leucosis is chronic and myeloid leucosis acute.

Inoculations with plasma were less successful than with blood but the incidence of erythro- and myeloid leucosis among the birds inoculated was sufficiently high (about 25 per cent*) to rule out spontaneous development as the sole possibility. The result of the individual experiments, however, were very variable; *e.g.*, in Series III-C injections with blood were almost 100 per cent positive but with plasma almost 100 per cent negative, suggesting, as with some viruses (F. W. Smith (23)), a relation of the agent transmitting leucosis to cells. In the earlier experiments the plasma was not recentrifugalized and may, therefore, have contained a very small number of cells. This negligence was due to the erroneous assumption that transmission was bound to a large number of viable cells. However, the positive

TABLE III
Incidence of Leucosis after Inoculating Various Material

Material used for inoculation	Total number of inoculated birds	Birds dead* from intercurrent disease	Birds surviving 30 days after inoculation	Results					
				Negative	M	E	ME	Ma	Ly
Whole blood.....	87	17	70	36	10	13	9	—	1
Blood cells.....	79	18	61	27	14	13	6	1	1
Fresh organ emulsion....	61	6	55	45	3	2	3	1	1
Plasma.....	43	11	32	22	3	3	2	2	—

* Within 30 days after inoculation.

inoculations with plasma recentrifugalized at high speed suggest that transmission may be accomplished by cell-free material. Indeed plasma passed through a Berkefeld filter that retained bacteria may occasionally contain the transmissible agent in sufficient concentration to cause leucosis in a high percentage of the inoculated animals. (See page 263.)

Ellermann mentions two experiments in which 16 fowls were inoculated, half with plasma and half with blood cells. Only two of the latter and one of the former developed leucosis. The description of this work is too incomplete for evaluation of the results.

Transmission by Organ Suspension.—Inoculations are less successful with organ suspension than with blood. Of the 41 birds inoculated

*Or 31 per cent if the two cases of myeloma are counted as positives.

IV-F	354M	W. B. C. 545,000 R. B. C. 1,115,000 Hb. 25	Blood cells	7	1	6	1	1	1	1
			Plasma	5		4				
			Berkefeld filtrate	8		7				
IV-J	365M	W. B. C. 320,000 R. B. C. 730,000 Hb. 16	Whole blood§	6		4				
			" "	4		3				
			Blood cells	7		3†				1
			Plasma	6	1	3				1
			Berkefeld filtrate	6		5				2
			Frozen and thawed cells	6	1	4†				2
			"Hemolysed" blood cells	6		4				1
V-G	485M	W. B. C. 506,000 R. B. C. 1,550,000	"Hemolysed" supernatant fluid	6		4			2**	
			Blood cells	3	1	2				
			Ground "Plasma"	5	3	1				
VI-B	798E	W. B. C. 42,500 R. B. C. 770,000 Hb. 15	Berkefeld filtrate	4	1	3		1††		
			Whole blood	4	3	1				
			Berkefeld filtrate, plasma	9		4				1
			" " cell extract	5		2		4		
				5		5		3		

All of these birds have been inoculated intravenously. The abbreviations are identical with those of Table I.
 † One of these might be incipient myeloid leucosis and erythroleucosis.
 § Reinjection of previously negative fowls.
 * Of these five were inoculated intravenously, two intramuscularly, and two intraperitoneally.
 ** One of these with sarcoma ovarii.
 †† Refers to one fowl (735) which had both myeloid leucemia and lymphoma.

The experiments reviewed briefly here tend to show that the difficulty in filtering the agent transmitting leucosis is chiefly technical. The factors influencing the outcome of filtration are not wholly understood; one of them appears to be similar to what is known to occur with the virus of vaccinia (F. W. Smith (23)), namely adherence to cellular material.

TABLE IV
Transmission Experiment from Fowl 798E

Number of fowl	Material used for inoculation	Amount inoculated	Result of the inoculation
		cc.	
874	Whole blood	0.5	Erythroleucosis, with recovery
875		0.5	Negative
878		0.5	"
885		0.5	Erythroleucosis
871	Whole blood	0.01	Negative
872		0.005	Erythroleucosis
870		0.0025	Negative
868		0.0012	Erythroleucosis
869		0.0006	Negative
877			
873	Plasma filtered through Berkefeld V candle	6.0	Erythroleucosis
876		4.0	Negative
880		4.0	Erythroleucosis
879		2.0	"
		3.0	Negative
883	Blood cell extract filtered through Berkefeld V candle	3.0	"
884		3.0	"
886		2.0	"
881		1.0	"
882		1.0	"

Among the 53 birds inoculated with Berkefeld filtrate in eight transmission experiments four chickens developed erythroleucosis, one myeloid leucosis, and two lymphoid leucosis.

In the first seven filtration tests an emulsion of the blood-forming organs was cleared by spinning and filtered through Berkefeld N (Series III-B, III-C, IV-B,

in ten series with organ suspensions, chiefly liver, spleen, and bone marrow, seven developed erythro- or myeloid leucosis.

Effect of Injury upon the Agent Transmitting Leucosis

The purpose of these experiments was to ascertain whether transmissions could be accomplished after injury resulting in destruction of leucemic cells (repeated freezing and thawing, and treatment with distilled water). Moreover rapid desiccation has been tried in attempts to preserve the hypothetical virus. Studies along these lines are being continued; those carried out simultaneously with the transmissions reported in this paper appear to be of sufficient interest to be summarized here.

Freezing and thawing did not seem to destroy the leucemia-transmitting agent, for two among twelve birds receiving such material developed leucosis.

These results are unlike those of Hirschfeld and Jacoby (1912) (12), who exposed material from organs of fowls with both leucosis and tuberculosis to a temperature of 10°C. below zero and on inoculation induced tuberculosis unaccompanied by leucemia.

Treatment with distilled water did not appear to be injurious to the leucemia-transmitting agent, for four of the ten fowls inoculated developed leucosis.

Desiccated organ suspension when tested on nine birds proved to be ineffective.

Experiments on the Filterability of the Transmissible Agent

Ellermann and Bang have maintained that the transmissible principle of the leucosis of the fowl is filterable. Their results have thus far not been confirmed, and the data presented by Ellermann in support of his opinion are not wholly conclusive. On the one hand he has included among his successful inoculations a variety of conditions whose relation to the transmissible agent is doubtful (*e.g.* lymphoid leucosis and simple anemia); on the other hand the possibility of a spontaneous leucosis was entirely disregarded by him. Andersen and Bang (1), who seem to have arrived at the same view, say that in a group of 14 fowls in one locality they have observed seven deaths caused spontaneously by leucemia during 1 year's observation.

ventionally under this heading are neither uniform nor do they appear to be connected with the transmissible erythroleucosis and myeloid leucosis. All the cases of lymphoid leucosis observed were extravascular, none of them appear to be primary in the marrow and the large lymphocytes of lymphoid leucosis could not be connected either with the formation of erythrocytes or of granulocytes.

Myeloma.—The following characters appear to differentiate this disease from myeloid leucemia: (a) A pronounced leucemic blood picture is not found although immature myeloid cells may be numerous in blood films; (b) the process does not appear to be primary in the bone marrow for although there is some hyperplasia of this organ it is not so diffuse and advanced as in myeloid leucemia; (c) in the liver myeloid nodules do not seem to favor the periportal area; (d) tumor nodes of similar structure may be found elsewhere, particularly attached to the bones. Some of these tumor nodes are composed purely of myelocytes, and may be designated myelocytoma.

The relation of myeloma to myeloid leucosis appears to be analogous to that of lymphoma to lymphoid leucosis. On account of this close relationship, one is inclined to interpret the cases of myeloma occurring in the experimental strain as caused by the transmissible agent. This assumption would seem to have been supported by the non-occurrence of similar conditions among the controls and by the occasional difficulty encountered in determining whether one is dealing with myeloid leucosis associated with myeloid tumors or with myeloma ending as myeloid leucemia (492, 481). On the other hand, it would seem from the study of Mathews (14), who has described similar spontaneous cases under the term leucochloroma, that this condition is not infrequent (0.5 to 1 per cent of the birds autopsied by him) and is not transmissible. Among our more recent passages not included in this report, myeloma did not occur and at the same time one spontaneous case was observed among a smaller number of uninoculated controls. Thus the relation of myeloma to transmissible myeloid leucosis remains unsettled until the observations shall have extended over a much larger number of birds than those reviewed here.

Tumors, Not Leucemic, Occurring in the Experimental Series

Three tumors, not leucemic, were observed among the fowls inoculated with material from myeloid or erythroleucosis and three among the controls. In passages of one of these (176, sarcoma) one case of lymphoid leucosis was observed among six fowls inoculated. One tumor (675, sarcoma) was associated with erythroleucosis and another (553) with myeloid leucosis. These and another similar spontaneous case gave rise to the thought that leucosis might be secondary to a tumor. The data presented, however, give no evidence that such tumors are produced by the transmissible agent described or that the

IV-F) or Berkefeld V filters (Series IV-J and V-G). In all of the filtration experiments the filtrates appeared to be clear and when inoculated on plain agar and broth proved to be sterile.

A more recent filtration experiment in Series VI-B with material from a fowl with severe erythroleucosis is fully presented in Table IV.

One group of five fowls were inoculated with plasma containing about 20 per cent heparin solution and passed through a Berkefeld V candle. The filtration lasted about 3 minutes, during which the pressure dropped to ± 4 cm. Hg. Another group of five fowls were inoculated with filtrate obtained from blood cells as follows. Cells from 18 cc. blood were gently ground in a porcelain mortar, taken up in isotonic salt solution, centrifugalized at low speed, and filtered through a Berkefeld candle V. *B. prodigiosus* added to the unfiltered material was retained completely by the filters. Erythroleucosis developed in three of the five fowls inoculated with 3 to 6 cc. of filtered plasma and the birds died of this disease from 42 to 62 days after inoculation. Among the fowls inoculated with whole blood, leucosis was about as frequent after inoculation with small amounts of blood (0.01 to 0.0006 cc.) as after inoculation with larger amounts of blood (0.5 cc.).

Fourteen fowls purchased from the same farm as those used in the above series of experiments were kept in the laboratory as "uninjected controls." None of them developed leucosis.

Summary of the Filtration Experiments.—The transmissible agent of the leucosis of fowls passes Berkefeld filters. Difficulties of filtration would appear to be chiefly technical, such as concentration of the transmissible material in the sediment when the blood or emulsion of organs is cleared from cellular material by spinning.

Leucosis of Which Causation by a Transmissible Agent Is Doubtful

There have occurred among the 377 birds used in the experimental series a variety of conditions that might be presented under the collective name leucosis, whose relation to the transmissible leucosis of fowls, however, is as yet unsettled. Some of these fall into the group of lymphoid leucosis and others might be collected under the term myeloma. It is desirable to differentiate them from those whose causation by the transmissible agent seems indisputable.

Lymphoid Leucosis.—It has been shown (page 253) that the transmissibility of lymphoid leucosis is thus far unproven and that it occurs as commonly among uninoculated as among inoculated fowls. The pathological processes grouped con-

cular. The relation of lymphoid leucosis to the transmissible agent has been discussed. (See pages 253 and 263.) Attempts at transmitting spontaneous lymphoid leucosis, which were unsuccessful, as were those of Andersen and Bang and Mathews and Walkey, will be described in a later report. It is noteworthy that in mammals (guinea pig, mouse) lymphoid leucosis has been successfully transmitted; a change of type, however, from the lymphoid to the myeloid has never been observed.

SUMMARY

Myeloid leucosis and erythroleucosis can be transmitted from one bird to others by emulsions of infiltrated organs, whole blood cells, and plasma. Inoculation is more often successful with blood cells or with whole blood than with plasma or with emulsions of organs infiltrated as the result of leucosis.

Inoculation with material from a bird with myeloid or with erythroleucosis produces both myeloid and erythroleucosis, and in many instances mixed forms with characters of both.

Evidence is wanting that lymphoid leucosis is caused by the agent that transmits myeloid and erythroleucosis. The occurrence of lymphoid leucosis among the birds inoculated with material from myeloid or erythroleucosis may be explained as spontaneous disease.

Injury to cellular structure by treatment with distilled water or by repeated freezing and thawing does not destroy the agent that transmits the disease.

Berkefeld filtrates have failed to transmit regularly myeloid or erythroleucosis. The evidence obtained shows, however, that the transmissible agent is filterable, although there are technical difficulties in its filtration.

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incidence of spontaneous neoplasms is increased after inoculations with transmissible leucemia.

DISCUSSION

Ellermann has described three types of leucosis of the fowl, lymphoid, myeloid, and erythroleucosis. Although in the present state of our knowledge there is some uncertainty whether the large cells resembling lymphocytes seen in lymphoid leucosis are actually lymphoblasts and whether the primitive lymphoid cells in erythroleucosis are actually erythroblasts, Ellermann's types appear to be distinct pathological conditions whose recognition is desirable to help clear the confusion on the subject of the leucosis of fowls.

Ellermann has succeeded in transmitting the leucemia of the fowl. The experience of Schmeisser, as well as our own, leaves little doubt as to the correctness of this conclusion. The idea of a filterable virus causing all three types of leucosis, however, is not well supported. On the one hand it has not been proved that the lymphoid type is transmissible at all, nor, on the other hand, that the agent transmitting myeloid and erythroleucosis is a virus.

The transmissible agent affects only a small percentage of inoculated fowls; some birds resist inoculation though they may receive several hundred times the amount of material necessary to produce leucosis in susceptible individuals. Resistance is not due to specific immunity (Ellermann). This behavior is unlike that of known viruses. That the transmissible agent sometimes passes bacteria-tight coarse or medium Berkefeld filters does not seem to constitute sufficient evidence for classifying it with the filterable viruses.

It has been shown that the transmissible agent resists procedures resulting in severe injury to animal cells, such as repeated freezing and thawing, treatment with distilled water. Further experiments on the nature of this agent are being continued in this laboratory.

In the bird, myeloid and erythroleucosis or a combination of these two conditions occur in passages of a single strain. The two processes are evidently intimately connected (page 247). For this reason Andersen and Bang proposed a collective term, "intravascular leucosis" for the transmissible leucosis of the fowl, but the term appears to be objectionable because myeloid leucosis is essentially extravas-

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the most striking feature of the microscopic examination consisted of an engorgement of the capillaries by lymphoid cells and an extreme hyperplasia of the bone marrow by similar cells.

Blood was drawn from this chicken into a chilled syringe containing a 1:1000 heparin solution, and within $\frac{1}{2}$ hour injected intravenously, in 1 cc. doses, into Chickens 51 to 63 inclusive (see Table I). Chickens 64 to 76 inclusive were controls.

Chicken 689 (passage IV-K) with the myeloid type of leukemia, had 310,000 white blood cells and 1,010,000 red blood cells per cubic millimeter. The differential count for white cells gave the following figures: large mononuclear and poikilonuclear cells, 63 (many of these distinctly of primitive type), similar cells with large purple granules (promyelocytes), 19, myelocytes, 8, polymorphonuclear leucocytes, 3, lymphocyte, 1, mononuclear cells with almost confluent vacuoles, 6. On gross examination the liver and spleen appeared to be normal; the bone marrow was pale, soft, and uniformly greyish. On microscopic examination the bone marrow showed an extreme myeloid hyperplasia; the blood was leucemic but extramedullary myeloid foci were not found.

Blood from this chicken was drawn into a chilled syringe containing a 1:1000 heparin solution and injected intravenously within $\frac{1}{2}$ hour into Chickens 77 to 88 (Table II) inclusive. Chickens 89 to 100 were left as controls. Each fowl was weighed monthly and a blood smear was taken from the controls each month, from each injected bird every 2 weeks, and more frequently from the affected fowls. All fowls that died were autopsied and sections were taken from the liver, spleen, and bone marrow.

The outcome of this experiment is shown in Tables I and II.

Tables I and II leave no doubt as to the transmission of leukemia for 5, or 36 per cent, of the first series and 9, or 75 per cent, of the second series developed either erythroleucosis or myeloid leucosis. One of the injected fowls developed lymphoid leucosis and one such case was found among the controls.

Of the five fowls in the erythroleucosis series that developed leukemia four had erythroleucosis and one myeloid leucosis. The four with erythroleucosis became pale and showed blood changes with many immature cells in from 30 to 60 days following injection; the first death occurred 48 days after injection. Another bird, a cockerel, No. 58, also became pale and developed a typical blood picture of erythroleucosis but later improved markedly and is still under observation. The myeloid case (No. 55) in the first series became pale in 42 days, died in 75 days, and was seen on postmortem examination to have an anemic carcass with pale red hemorrhages in the muscles. The liver

TRANSMISSION EXPERIMENTS WITH LEUCOSIS OF FOWLS

By E. L. STUBBS, V.M.D., AND J. FURTH, M.D.

(From the School of Veterinary Medicine and The Henry Phipps Institute of the University of Pennsylvania, Philadelphia)

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In connection with the work at The Henry Phipps Institute on leucemia of fowls, it seemed desirable to continue outside of the Institute some transmission experiments with the same stock of birds, so planned as to control carefully the spontaneous occurrence of leucemia. The similarly planned experiments begun at the Institute had been broken off by an epidemic of intercurrent disease. Those to be reported here were performed at the Veterinary School of the University of Pennsylvania, where no transmission experiments in leucemia had been done heretofore.

Fifty young Barred Plymouth Rock chickens were purchased from the same source as the chickens used at The Henry Phipps Institute. They were about equally divided as to sex and weighed from 710 to 1000 gm. Each chicken was legbanded and a blood smear taken before the beginning of the experiment. They were placed in cages holding four or five birds each in two stands and arranged vertically, in stacks of three. The fowls in one stack were injected and those in the next stack of three cages left uninjected, giving an equal number of injected birds and of controls arranged, horizontally, in three tiers of four alternating cages.

Two fowls with the transmissible leucemia strain of The Henry Phipps Institute, one having an erythroleucosis and the other a myeloid leucosis, served as donors. The former, No. 575, (passage IV-G) had 95,000 white blood cells and 770,000 red blood cells per cubic millimeter of blood. In blood smears numerous erythroblasts were seen; many of these were of the immature type, as indicated by the strong basophilia of the cytoplasm and apparent lack of hemoglobin. A considerable percentage of the leucocytes were doubtless lymphoid cells with erythroblastic potencies. Among 100 white cells there were about 20 lymphoid erythroblasts and about 29 primitive lymphoid cells, presumably precursors of erythroblasts; the rest were polymorphonuclear leucocytes, 15, myelocytes, 3, large mononuclear cells, 18, and small lymphocytes, 15. Paleness of the organs and splenomegaly were the most notable observations on gross examination, and

Incidence of Leucosis in Fowls Injected March 17 with Blood from Fowl with Myeloid Leucosis (No. 689) and in Controls

Chicken No.	Weight, March 17	Weight, April 17	Weight, May 14	Weight, June 11	Weight at death	D. = died K. = killed	Weight of liver	Weight of spleen	Blood smear	Result	
Injected											
77	720					D. April 7	25	4	Negative	—	Tumor resem- bling Rous sarcoma
78	789	1250	1475	1975	1920	K. June 23	50	5	"	—	
79	920	1250	1060		1100	D. June 11	52	8	Erythro	+	
80	830	1250	1170		1070	D. May 17	80	20	Myeloid	+	
81	750	1080	950	1375					Erythro	+	Kept alive for further obser- vation
82	870	1120	770		700	D. May 22	30	7	"	+	
83	930	1350	900		1020	D. May 20	80	20	"	+	
84	630	1020			632	D. May 28	55	15	"	+	
85	820	1050			1050	D. April 21	73	10	"	+	
86	880	1250			1260	D. April 22	30	4	Negative	—	
87	910	1180	1410	1750	1575	D. June 13	125	27	Erythro	+	
88	830	1080				D. May 16			"	+	
Controls											
89	940	1320	1600	1995	2010	K. June 23	50	5	Negative	—	
90	870	1320			1300	D. May 5	25	4	"	—	Fracture of the femur
91	830	1020	1270	1575	1530	K. June 23	45	4	"	—	
92	830	1020	1270	1575	1210	K. June 23	35	4	"	—	
93	700	950	1100		905	D. May 20	70	15	"	—	Aluecemic lymph- oid leucosis
94	950	1410	1750	1995	1980	K. June 20	44	5	"	—	
95	950	1050	1300	1245	1100	K. June 23	35	4	"	—	
96	730	1170	1300	1730	1710	K. June 23	50	4	"	—	
97	710	1080	1250		935	D. June 2	50	7	"	—	
98	710	1100	1250	1495	1340	K. June 23	30	4	"	—	
99	950	1360	1500	1645	1680	K. June 23	35	4	"	—	
100	910	1400	1650	1995							Kept alive for further obser- vation

TABLE I

Incidence of Leucosis in Fowls Injected March 17 with Blood from Fowl with Erythroleucosis (No. 575) and in Controls

Chicken No.	Weight, March 17	Weight, April 17	Weight, May 14	Weight, June 11	Weight at death	PD = died = killed	Weight of liver	Weight of spleen	Blood smear	Result	
Injected											
51	920	980	1370		1190	D. June 6	215	3	Negative	-	Aleucemic lymphoid leucosis
52	810	1070	1250	1650	1804	K. June 19	40	4	"	-	
53	1000	1400	1600	1850	1984	K. June 19	38	5	"	-	
54	770	1050	1250	1650	1784	K. June 19	45	5	"	-	
55	790	970	1195		903	D. June 2	60	13	Myeloid	+	Kept alive for further observation
56	960	1340	1650		1380	D. May 2	154	37	Erythro	+	
57	750	1350	1470	1700	1800	K. June 19	39	6	Negative	-	
58	880	1350	1395	1600					Erythro	+	
59	730	1150	1420	1550	1630	K. June 19	38	5	Negative	-	
60	780	1250	1500	1550	1670	K. June 19	38	5	"	-	
61	750	1200			900	D. May 5	80	15	Erythro	+	
62	990	1500	1700	1900	2000	K. June 19	41	5	Negative	-	
63	830	1050			783	D. May 12	73	15	Erythro	+	
Controls											
64	810	1280	1376	1660	1750	K. June 20	31	5	Negative	-	Kept alive for further observation
65	910	1300	1500	1500	1790	K. June 20	40	4	"	-	
66	830	1270	1570	1920	1920	K. June 20	50	5	"	-	
67	790	1250	1270	1470	1620	K. June 20	40	5	"	-	
68	810	1150	1370	1670					"	-	
69	880	1350	1670	1800	1615	D. June 17	75	5	"	-	
70	870	1350	1670	1800	1810	K. June 20	36	4	"	-	
71	820	1050	1200	1895	1895	K. June 20	37	5	"	-	
72	910	1250	1550	1615	1690	K. June 20	34	5	"	-	
73	800	1000	1150	1425	1520	K. June 20	38	5	"	-	
74	980	1300	1530	1710	1820	K. June 20	50	5	"	-	
75	880	1100	1400	1485	1530	K. June 20	50	5	"	-	
76	840	1100	1350	1445	1570	K. June 20	30	6	"	-	

large mononuclear cells (myeloblasts, Ellermann), several of which were in mitotic division. Only a few myelocytes were seen. The red blood cell count was 1,300,000 and the white cell count 800,000. Several of the red cells were polychromatophile and numerous erythroblasts were found. Two weeks later, on June 11, the abnormal white cells had practically left the circulation and the total number of white cells appeared to be below normal. Erythroblasts were numerous and several were immature.

Table II shows the outcome of the transmission from Fowl 689 (myeloid leucosis). Of 12 injected fowls, 9 (75 per cent) developed either erythroleucosis (8 cases) or myeloid leucosis (one case). These birds showed paleness and blood changes in 30 to 72 days and died during the period from 34 to 88 days following injection.

As in the first series, postmortem examination of seven of these leucemic fowls showed enlarged livers and spleens. The livers weighed from 30 to 125 gm. and were of a reddish brown color and the spleens weighed from 7 to 27 gm. Comparison of average weights showed increase in these organs and loss of total body weight as well, with an average weight of carcass, in the affected birds, of 1021 gm., of liver, 71 gm., and of spleen, 15 gm., and an average weight in the controls of 1540 gm. carcass, 38 gm. liver, and $4\frac{2}{3}$ gm. spleen.

No. 77 died, 21 days following injection, with a tumor in the thigh region, which on gross and microscopic section resembled a Rous sarcoma. The blood-forming organs presented no leucemic involvement.

No. 81 showed 4 weeks after injection numerous erythroblasts in the circulation with a few cells of the lymphoid type and the picture of distinct but mild erythroleucosis. 2 weeks later only occasional erythroblasts were seen and smears taken subsequently at 2 weeks' intervals were normal.

No. 90, one of the controls, became very lame, was killed, and showed a fracture of the femur. Postmortem examination, blood examination, and microscopic sections of liver, spleen, and bone marrow were all negative for leucemia.

No. 98, also a control, showed an enlarged liver and spleen. A blood smear taken 6 days before death gave the following figures in differential counting: small lymphocytes, 80, mononuclears, 7, polymorphonuclears, 10, mast cells, 3. The number of white cells did not seem to be increased. Microscopic sections showed lymphoid leucosis.

No. 97, a control, died. Microscopic appearances of this case fall within the definition of lymphoid leucosis and the picture was unlike that seen in injected fowls that developed erythro- or myeloid leucosis. A blood smear taken 4 days before death showed increased number of white cells; most of these were medium-sized cells resembling lymphocytes and large mononuclear leucocytes. The bone

was slightly enlarged, weighing 60 gm., and the spleen was enlarged to twice its normal size, weighing 13 gm.

The four fowls with erythroleucosis all lost weight and averaged at death only 991 gm., whereas the 12 controls averaged 1711 gm. when killed. On postmortem examination they showed many pale red hemorrhages in various locations, particularly over the humeral region and over the knee joint. The livers were all slightly enlarged, had a reddish brown color, and weighed from 73 to 154 gm. The average weight of the liver was 82 gm., and in the 12 controls 42 gm., or less than one-half the former. The spleens were estimated to be from two to six times the normal size, weighing 15 to 37 gm. and averaging 20 gm. or four times the average weight of 5 gm. in the 12 controls.

Similarly when the erythroleucosis cases are compared with the controls together with the injected fowls that did not develop leucemia, a marked difference in weight is found. The averages in the leucemic birds, namely, carcass 991 gm., liver 92 gm., and spleen 20 gm., contrasts with the following averages in all the other fowls of this series except No. 555, described above, and No. 51, described below, namely, carcass 1760 gm., liver 40 gm., and spleen 5 gm. From these data it appears that the injected fowls that did not develop leucemia showed no loss of weight.

No. 51 was a case of lymphoid leucosis with blood smear and microscopic examination of liver, spleen, and bone marrow showing no indication of leucemia. Postmortem examination revealed an enormously enlarged liver, weighing 215 gm., or one-fifth of the weight of the entire carcass. The spleen weighed only 3 gm. The liver showed many areas from pin-head size to $\frac{1}{2}$ inch across, yellowish grey in color, giving it a mottled appearance, and microscopic section showed masses of cells resembling lymphocytes without any blood alteration. Hence the pathological condition is diagnosed lymphoid leucosis. Aleucemic lymphoid leucosis was also found in one control fowl; it is, therefore, doubtful that the injection had anything to do with this case.

No. 58 of this series is of particular interest. On May 1, 1930, about 6 weeks after injection, several erythroblasts, including many younger forms, appeared in the circulation, but polychrome red cells were practically absent. 14 days later the number of erythroblasts showed considerable increase, the number of white cells appeared to be moderately increased and composed of large mononuclears, large primitive cells, polynuclears, and a few myelocytes. On May 28, 14 days later, the circulating blood appeared to be flooded by large poikilonuclears and

SUMMARY AND CONCLUSIONS

It is shown, in a carefully controlled experiment, that fowl leucemia can be readily transmitted from chicken to chicken by injection.

Of 25 fowls, 13 were injected intravenously with blood from a chicken (No. 575) with erythroleucosis and 12 with blood from a chicken (No. 689) with myeloid leucosis. The injected birds and an equal number of uninjected controls were kept in tiers of alternating cages under as nearly identical conditions as possible. The donors, the controls, and the injected birds were all of the same stock and of the same age.

In the two series 13 or 52 per cent of the 25 fowls inoculated developed leucemia within 4 to 10 weeks after injection. The two types of leucemia, erythroleucosis and myeloid leucosis, developed in both groups irrespective of the type used for injection. Among the uninjected controls no cases of erythroleucosis or of myeloid leucosis were observed. Lymphoid leucosis occurred in one injected fowl but since it also occurred in one control it may be assumed that it was probably not caused by the injected material.

marrow was moderately fatty and showed a mild granulocytic hyperplasia. The liver contained numerous foci of infiltration with cells like lymphocytes, chiefly around the vessels. The content of larger vessels did not seem to be leucemic. In capillaries, however, which were congested, cells like lymphocytes appeared to be somewhat increased in number.

DISCUSSION

A knowledge of the incidence of spontaneous leucosis would seem essential to control the results of the transmission experiments. So far as we know this is the first systematically controlled experiment, in an attempt to transmit leucemia of fowls, in which a number of birds were used as controls equal to the number injected, both groups being from the same source and kept side by side under as nearly identical conditions as possible.

The experiment shows clearly that leucemia of fowls can be transmitted from fowl to fowl by injection, and with a fairly high percentage of successful inoculations when blood is injected intravenously. Of twenty-five fowls inoculated, 13 or 52 per cent developed leucemia.

Two types of leucemia occurred among the injected fowls, one in which the blood picture showed large numbers of erythroblasts and the other with large numbers of large mononuclears and myelocytes. In the former, the capillaries are crowded with lymphoid cells, which are assumed to be precursors of erythroblasts as suggested by Ellermann. The bone marrow shows in both types an extreme hyperplasia formed by cells similar to those seen in the capillaries. Hence we have applied the terms erythroleucosis and myeloid leucosis of Ellermann to the two conditions respectively.

We are unable to differentiate the two types with certainty by the gross postmortem appearance of the organs, and the method of differentiation was based on the blood examination before death and on the microscopic changes in the liver, spleen, and bone marrow after death. The two types appear to be closely related, and the one or the other appears to develop independently of the type used for injection.

Postmortem examination of the fowls with the transmitted leucemia did not show the enormous liver that is found in the rather frequent condition popularly termed "big-liver disease" and sometimes called leucemia. This apparently is an aleucemic condition and falls within the type lymphoid leucosis of Ellermann's terminology.

TABLE I
Showing Source and Kind of Material Used

Swine No.	Route of infection	Virus strain	Nervous symptoms	Killed (k.) or died (d.) after infection	General pathological and anatomical diagnosis	Histological lesions			Remarks
						Encephalitis	Menigitis	Hemorrhages	
1	Intramuscular	1	+	6 (k.)	No typical hog cholera	+	+	+	
2	"	1	-	7 "	"	-	-	-	
3	"	1	+	8 "	Typical hog cholera	++	+	-	
4	"	1	?	8 "	"	++	+	+	
5	"	1	?	8 "	"	+	+	-	
6	"	2	-	8 "	"	++	++	-	
7	"	2	-	8 "	"	+	+	-	
8	"	2	-	8 "	"	++	+	-	Very slight lesion
9	"	1	++	8 "	"	-	-	+	
10	"	1	++	9 "	"	±	-	-	
11	"	1	+	9 "	"	-	-	-	Congestion of blood vessels
12	"	1	?	9 "	"	++	+	+	
13	"	3	+	9 "	"	+	+	-	
14	"	3	?	9 "	"	±	-	-	
15	"	1	+	9 "	"	++	+	-	
16	"	1	?	10 (d.)	"	++	++	-	
17	"	1	++	11 (k.)	"	++	++	+	Marked infiltration
18	Contact	3	+	12 "	"	++	+	+	
19	"	4	+	12 "	"	++	-	-	
20	"	1	?	13 "	"	++	+	+	
21	"	2	+	13 "	"	++	+	-	Glia proliferation
22	"	3	?	13 "	"	++	+	+	Many glia nodules
23	"	1	-	14 "	No typical hog cholera	±	-	-	

HISTOLOGICAL STUDIES ON HOG CHOLERA

I. LESIONS IN THE CENTRAL NERVOUS SYSTEM*

BY OSKAR SEIFRIED,† V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

PLATES 15 TO 17

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Although symptoms that can be referred to the central nervous system have been observed clinically in hog cholera (1, 2, 3), papers dealing with the anatomical lesions are few and far from complete. Von Hutya and Marek (2) state that the spasms, convulsions, staggering, and lethargy are due to hemorrhages in the meninges and brain substance. They further note that convalescent animals may suddenly die with symptoms of brain hemorrhage. Huguenin (4) found hemorrhages, edema, hyperemia, and inflammatory processes in various degrees in about 20 per cent of the cases studied. Later Brunschwiler (5) studied the central nervous system in various swine diseases. Among these were seven cases of hog cholera in which hyperemia, edema, hemorrhages in the meninges, and meningitis were found. In one case there were inflammatory lesions in the brain substance. He collected data on 61 cases of hog cholera, some of these cases being from the literature, and showed that brain lesions were present in 24 cases, or 39.3 per cent.

In the descriptions of lesions of the central nervous system that have been published there are no notes concerning the glia and nerve cells, and the distribution of the inflammatory reaction has not been studied. It is quite possible also that some of the cases were not virus hog cholera.

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† Professor extraordinary at the University of Giessen, Germany.

Material

The material used for this investigation consisted of 39 cases of hog cholera that were used in other experiments on this disease. The animals were infected by intramuscular injection or by contact. Some were killed with chloroform in 6 to 49 days after infection while others died. Four different strains of virus were used; two were laboratory strains that had been passed artificially for a considerable period of time, and two were strains from fresh outbreaks in the Middle West. All infected animals had a characteristic temperature, and the majority showed what are regarded as typical lesions at autopsy, *i.e.*, hemorrhages in the kidney, lymph glands, oftentimes in the bladder and large intestine. Many of them developed central nervous system symptoms of varying degree. Seven normal pigs from the same source as the infected ones were used as controls. In Table I are summarized the more important data relating to these animals.

Methods

As noted above, chloroform was used for killing the animals and autopsy was done immediately after death. Most of the tissues from the central nervous system were fixed in 10 per cent formaldehyde. In some cases formaldehyde was injected through the carotid arteries in order to fix the entire brain. For special neuropathological methods, Zenker's and Mueller's fluids, alcohol, and bromformol were used for fixation. Hematoxylin and eosin and van Gieson and Mallory's methylene blue phloxin were used as routine stains. The following were used for special purposes: Nissl's stain, Spielmeyer's and Kulschitzky's stains for myelin-sheaths, Bielschowsky's stain for axis-cylinders, stains for glia fibers (Alzheimer-Mann, Heidelberg, Oppenheim, iron-hematoxylin), stains for neurofibers (Bielschowsky, Cajal); and furthermore the glia methods of Rio del Horteaga and Cajal, the stains with methylgreen-pyronin, Weigert's fibrin and elastic tissue stains, iron and oxydase reactions, fat stain with Scharlach R and osmic acid, Klarfeld's tannic acid and silver method, Giemsa's stain, and Mann's, Lentz's, Stutzer's, and Hammerschmidt's stains for inclusion bodies.

Gross Pathology

Macroscopic changes of the central nervous system are not characteristic, except for a more or less marked congestion of the blood vessels. In a few cases pronounced hemorrhages and edema in the meninges, sometimes covering large areas of the brain and spinal cord, and in the brain substance are present. In such cases the liquor cerebri shows a slight yellowish red or red color.

	Intramus- cular	1	?	15 "	Typical hog cholera	++	++	+	Numerous glia nodules
24									
25	"	1	++	16 (d.)		++	++	+	Very slight lesions
26	"	1	++	16 "		++	++	++	Slight encephalitis, hemorrhages pre- dominating
27	"	1	?	17 "		++	++	++	Marked hemorrhages in brain and spinal cord
28	"	1	++	17 (k.)		-	-	++	Pons and medulla oblongata espec- ially involved
29	"	1	++	19 "		++	++	+	Marked lesions in the spinal cord.
30	"	1	++	20 "		++	++	+	Some necrotic foci
31	"	1	++	20 (d.)		++	++	++	Clotted blood between dura and pia mater
32	"	1	++	20 (k.)		++	++	++	Marked hyperemia
33	"	1	++	20 "		++	++	++	"
34	"	1	++	24 "		++	++	++	Many glia nodules
35	"	1	+	29 "		++	++	++	
36	"	1	++	33 "		++	++	++	
37	"	2	+	34 (d.)		++	++	++	
38	"	3	?	35 "		++	++	++	
39	"	1	++	49 "		++	++	++	

Controls					Previously infected with swine influenza, without developing the disease				
40	-	-	-	Normal	-	-	-	-	-
41	-	-	-	"	-	-	-	-	-
42	-	-	-	"	-	-	-	-	-
43	-	-	-	"	-	-	-	-	-
44	-	-	-	Spontaneous enteritis	-	-	-	-	-
45	-	-	-	Normal	-	-	-	-	-
46	-	-	-		-	-	-	-	-

accumulations of cells the nature of which, whether glial or mesodermal, will be discussed later. Other changes in the vessels are hyperemia, swelling and degeneration of the endothelial cells, perivascular edema with suppuration of the connective and elastic tissue fibers, and more or less distension of the submarginal glia spaces. The formation of new capillaries has not been found in the inflammatory areas.

In addition to the above lesions there have been seen in a number of cases blood vessels in the brain and meninges that do not show perivascular infiltration but that are surrounded by a thin wall of red blood corpuscles. Similar microscopical hemorrhages have also been seen in the tissues unassociated with vessels (Fig. 1). It is worthy of note, however, that hemorrhages in the central nervous system are relatively infrequent while in other parts of the body they seem to be the predominating lesion. When found they are more pronounced in the cerebellum and spinal cord and they may be extensive enough to be seen with the naked eye.

Perivascular infiltrations may be found in the choroid plexus (Fig. 6) and the cerebrospinal fluid may contain mononuclear cells and red blood corpuscles, especially when there are hemorrhages in the meninges. Thrombi, emboli, and patches of softening have not been observed. In one case necrotic foci have been found, but these are apparently due to secondary invaders, for bacteria are present in great numbers in the capillaries and smaller vessels.

(b) *Lesions of the Glia.*—In hog cholera just as in Borna disease, dog distemper, and rabies, there is a more or less marked proliferation of the microglia and neuroglia which may be a prominent feature in the process. These glia proliferations vary according to the duration and probably also to the severity of the disease. The most common picture consists of small nodules often seen in the white matter, which are rather sharply outlined against the surrounding tissues (Fig. 7). In other cases there is a more diffuse proliferation of the glia cells (Fig. 8). In some cases these lesions are made up only of proliferating glia cells while in others mononuclear cells may also be found (Fig. 9). The latter type of lesions has been observed chiefly in the neighborhood of blood vessels, the walls of which are infiltrated with mononuclear cells. It has also been observed under the ependyma of the ventricles which

Histology

The histological lesions may be divided into inflammatory and degenerative changes. They will be discussed under the headings of (a) mesodermal tissue, (b) glia, and (c) nerve cells.

(a) *Lesions of the Mesodermal Tissue.*—The most obvious and striking microscopical change is the mononuclear infiltration of the perivascular spaces of the parenchyma of the brain and spinal cord (Figs. 1 and 2). In the majority of cases the lesions in the brain substance are more marked than those in the meninges. We feel therefore that we are not dealing with a so-called "meningo-encephalitis" in which the infection spreads inward from the meninges, but that we have a true encephalomyelitis with a secondary involvement of the meninges (Figs. 3 and 4). The degree of perivascular infiltration which involves the smaller veins and arteries varies greatly. In early stages it may be slight or even absent, while in older cases regular perivascular "cuffings" with several layers of cells may be found. In some cases these cuffings are so large that they may be seen in stained sections with the unaided eye (Fig. 1). In an individual case the degree of infiltration varies and apparently is dependent upon the location of the area in the central nervous system. In general the lesions are more comparable with epidemic encephalitis in man than with Borna disease of horses in which the infiltration is more extensive.

The infiltrating cells consist largely of small lymphocytes and mononuclear elements, which are variously called polyblasts or macrophages, with a certain number of plasma cells and occasionally a few eosinophilic leucocytes. Polymorphonuclear cells are absent and the oxydase reaction is negative. Mitotic figures, fragmentation of nuclei, and other products of degeneration of the infiltrating cells are frequently found. The macrophages contain lipoids in varying amounts. Granulo-adipose cells with the form of a mulberry may be quite numerous. Iron pigment in the macrophages has been found only occasionally.

When the lesions are comparatively slight Klarfeld's tannic acid and silver method shows that the infiltrating cells remain limited to the membrana glia limitans (Fig. 5). In the more extensive lesions there are found in the immediate neighborhood of the involved vessels,

Schweinsberger disease in horses, characterized by marked hypertrophy of the nucleus and protoplasm, have been found. Sometimes three to six of these hypertrophied cells with their protoplasm connected have been demonstrated. Retrogressive changes are less common and are most frequently seen in cells which have previously shown the above lesions. They consist of degeneration of the nuclei and are characterized by partial or total hyperchromatosis, pycnosis, atrophy, etc. Karyorrhexis and fragmentation of the nuclei are seen only occasionally.

The inflammatory lesions, *i.e.*, the perivascular infiltration and the glia proliferation, have been seen in both the white and gray matter and there seems to be no special selection of the gray substance as in various types of encephalitis. Our preliminary studies show that the type of lesion as well as the distribution in hog cholera and dog distemper are quite similar (6).

(c) *Lesions of the Nerve Cells.*—The nerve cells show changes in the areas involved by inflammatory processes and also in parts of the central nervous system that are comparatively free from other lesions. The degree of change does not seem to be dependent upon the severity of the inflammatory process. Nerve cells in one area may show marked changes while adjacent cells may appear to be normal. In exceptional cases the large pyramidal cells seem to be involved more severely than the multipolar cells of the cerebral cortex and gray nuclei.

After examining a large number of preparations we feel that the changes found in the nerve cells are not diagnostic. They are chiefly of the degenerative type described by Nissl as “akute Zellerkrankung” and “schwere Zellveränderung,” and are characterized as follows. The nucleus in an individual cell is swollen, is located peripherally, the chromatin is fragmented and the nucleolus absent. The protoplasm shows more or less marked tigrolysis; it contains vacuoles, the cellular membrane is denticulated, and Nissl’s granules may be lost, so that only cell shadows are present. These lesions may be found in the cells of Purkinje but the so-called “homogenisierende Zellerkrankung” of Spielmeyer has been seen only rarely in these cells. The neuronophagia and glia nodules referred to in the previous section are often associated with this kind of cell degeneration.

The methods of Bielschowsky and Cajal have failed to reveal a

otherwise appear unchanged. Frequently a mobilization of neuroglia elements composed chiefly of cells of the Hortega type may be found around infiltrated vessels where they form areas having a peculiar circular arrangement. This is brought out in Fig. 10. In the areas of neuroglia proliferation mitotic figures are sometimes very numerous.

Satellitism is met with frequently in the gray matter of the brain, cerebellum, and spinal cord, and represents an increase of glia elements around ganglion cells. In some cases neuronophagia has been observed, as shown in Fig. 12. So-called "glial stars" or "glial rose-knots" which are present in the gray matter may be a later stage of this process. Spielmeyer's "glial shrub-wood" had been found in some cases in the molecular zone of the cerebellum but not in a typical form.

Glia nodules were first described in rabies by Babes and are usually known as "nodules of Babes." For some time they were considered to be specific for this disease but it is now known that they appear in other types of encephalitis in man and animals.

The methods of Cajal and Rio del Hortega show that both the microglia and macroglia are involved in the proliferation process. The demonstration of microglia proliferation by Hortega's method was more difficult in swine than it is in rabbits. We have the impression that the microglia predominates in the acute and subacute stages of the disease while in the more chronic cases the macroglia cells are more noticeable. The latter are found exclusively in the proliferating areas surrounding the third and the fourth ventricle while accumulations near infiltrated vessels consist largely of microglia cells. Bacilli-like cells, or "Stäbchenzellen," which probably represent special types of microglia cells, have been found rather frequently.

There is very little increase of glia fibers, the nodules and other lesions being made up largely of the protoplasmic part of the glia cells. In some older cases a slight increase of glia fibers surrounding infiltrating vessels could be demonstrated (Fig. 11) but this is the exception rather than the rule. The increase of glia cells in hog cholera is not as prominent as in Borna disease or in epidemic encephalitis of man.

Lesions of glia cells resembling those in pseudosclerosis in man and

cytes, mononuclear elements, a few plasma cells, and occasionally a few eosinophilic leucocytes. The glia shows a proliferation surrounding infiltrated vessels or forming small nodules or more diffuse foci. Satellitism and in a few instances true neuronophagia have been observed. Both microglia and macroglia participate in this process. There is no essential increase of glia fibers. In nearly all parts of the central nervous system degenerating lesions of the nerve cells such as tigrolysis and degeneration of the nucleus, including a slight atrophy of endocellular neurofibers, are encountered. No demyelination has been observed. Specific inclusion bodies in the nerve cells are absent. In addition, in a certain number of cases microscopic and macroscopic hemorrhages are present in the brain, spinal cord, and meninges.

3. These lesions in varying degrees have been found in swine infected with four different strains of hog cholera virus. Two were laboratory strains and two were obtained from fresh field outbreaks.

4. Histological changes in the central nervous system were found as early as 6 days after infection before the animal showed central nervous system symptoms. In two cases which were paralyzed no lesions in the central nervous system could be demonstrated.

5. The lesions in the central nervous system are considered to be the anatomical substratum for the various nervous symptoms commonly found in hog cholera.

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hypertrophy of the endocellular neurofibers such as is found in rabies and distemper. They show, on the other hand, an atrophy and fragmentation and a type of perinuclear accumulation. The extracellular fibers stain normally or may at times be slightly hypertrophied. Demyelination in the parenchyma of the brain such as has been found in dog distemper by Perdrau and Pugh (7) has not been demonstrated in our sections. Sections of the spinal cord stained by Marchi's method show a degeneration of individual fibers throughout the white matter (Fig. 14).

Inclusion Bodies.—Uhlenhuth and his collaborators have found in the corneal epithelium in swine infected with hog cholera virus bodies simulating inclusions. According to a statement of Huguenin (4) inclusions could not be demonstrated in a variety of tissues including the central nervous system. Careful examination of our material by various methods has failed to reveal typical inclusion bodies in the central nervous system. In a number of cases intranuclear bodies resembling inclusions have been found in the nerve cells of various parts of the brain. In some instances they have been acidophilic, homogeneous, round, and sometimes surrounded by an unstained halo resembling closely Joest-Degen's inclusion bodies in Borna disease (Fig. 13). They have been found singly or in groups up to five in number. Their dimensions have varied from the limit of visibility to the size of a nucleolus. Most of them, however, take a basophilic stain, being somewhat lighter in color than the nucleus. Bielschowsky's method has revealed a sort of structure with argento-philic properties. These bodies have been found in only a small percentage of cases, and since they do not as a rule show the staining properties of inclusion bodies, we regard them as products of nuclear degeneration rather than true inclusions.

SUMMARY AND CONCLUSIONS

1. A more or less marked encephalomyelitis and meningitis was found in 33 out of 39 cases of virus hog cholera which had been infected either intramuscularly or by contact and killed between 6 and 49 days after infection.
2. This hog cholera encephalitis is characterized by a varying amount of vascular and perivascular infiltration with small lympho-



EXPLANATION OF PLATES

PLATE 15

FIG. 1. Medulla oblongata. Perivascular "cuffings," infiltration of the tissue, glia proliferation, hemorrhages. Hematoxylin-eosin. Swine 32. $\times 35$.

FIG. 2. Midbrain. Perivascular infiltration and glia mobilization surrounding the aqueduct. Nissl stain. Swine 12. $\times 85$.

FIG. 3. Cortex of cerebellum. Marked meningitis. Sulcus. Hematoxylin-eosin. Swine 32. $\times 35$.

FIG. 4. Midbrain, cross section through corpora quadrigemina. Marked meningitis and encephalitis. Hematoxylin-eosin. Swine 32. $\times 35$.

FIG. 5. Wall of an infiltrated vessel showing infiltrating mononuclear cells between the separated and extended connective tissue fibers. Klarfeld's tannic acid silver method. Swine 25. $\times 570$.

PLATE 16

FIG. 6. Choroid plexus. Congestion and perivascular infiltration of the blood vessels. Hematoxylin-eosin. Swine 21. $\times 60$.

FIG. 7. Neighborhood of third ventricle. Glia proliferation forming a so-called glia nodule. Nissl stain. Swine 21. $\times 145$.

FIG. 8. Midbrain, substantia nigra. Diffuse glia proliferation, with many cells of the Hortega type, in the surroundings of ganglion cells. Nissl stain. Swine 22. $\times 100$.

FIG. 9. Immediate neighborhood of third ventricle. Diffuse proliferation, mononuclear infiltration, and hemorrhages. Hematoxylin-eosin. Swine 39. $\times 100$.

FIG. 10. Midbrain, surroundings of aqueduct. Infiltrated vessel with perivascular glia proliferation. Nissl stain. Swine 25. $\times 85$.

PLATE 17

FIG. 11. Infiltrated vessel with extension of submarginal glia spaces and slight increase of glia fibers. Iron-hematoxylin. Swine 17. $\times 660$.

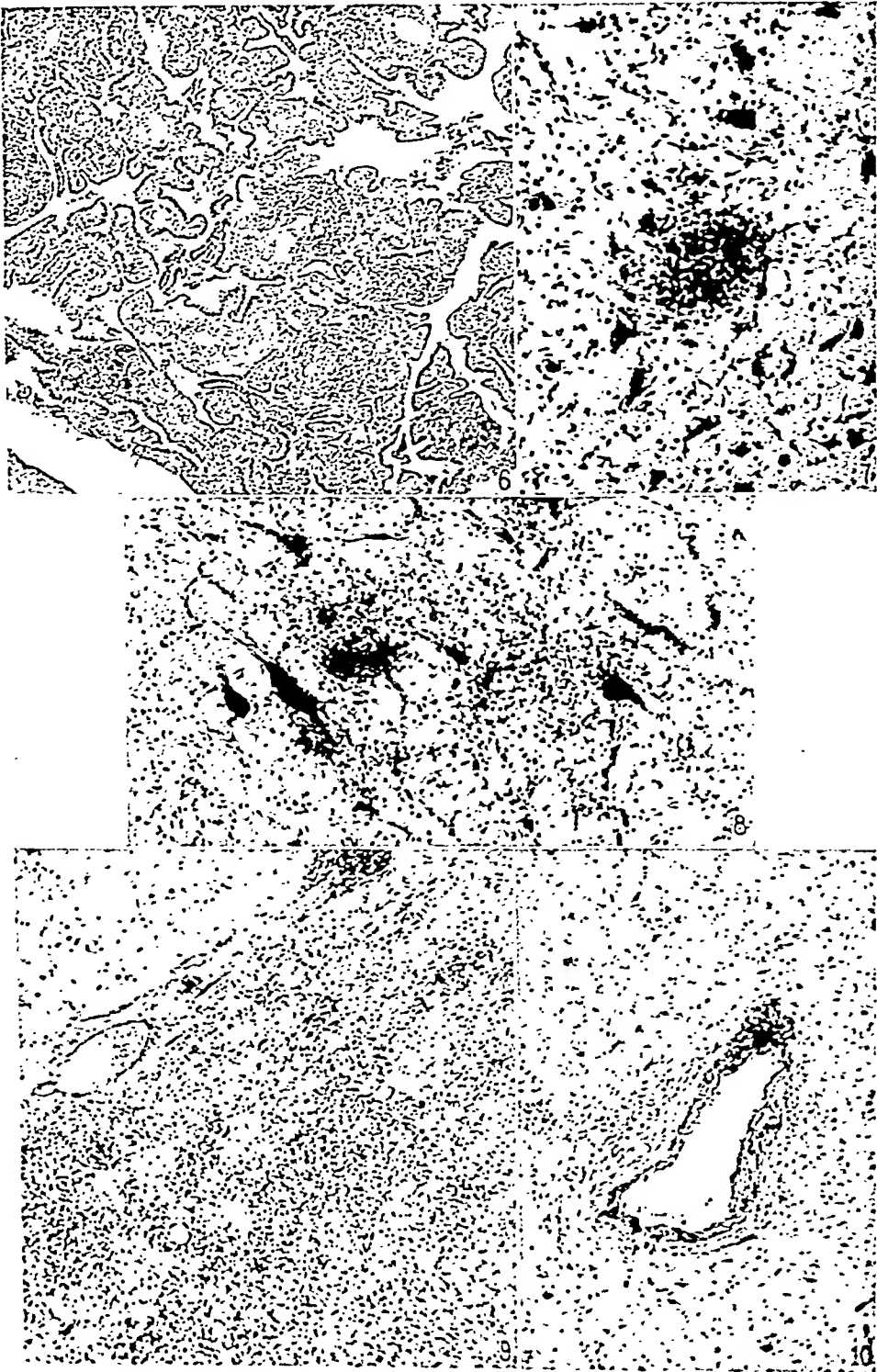
FIG. 12. Interbrain, neighborhood of third ventricle. Neuronophagia. In the center of the cell accumulation a degenerating nerve cell. Nissl stain. Swine 21. $\times 660$.

FIG. 13. Medulla oblongata. Degenerating nerve cell with an intranuclear body simulating an inclusion body. Giemsa stain. Swine 21. $\times 1000$.

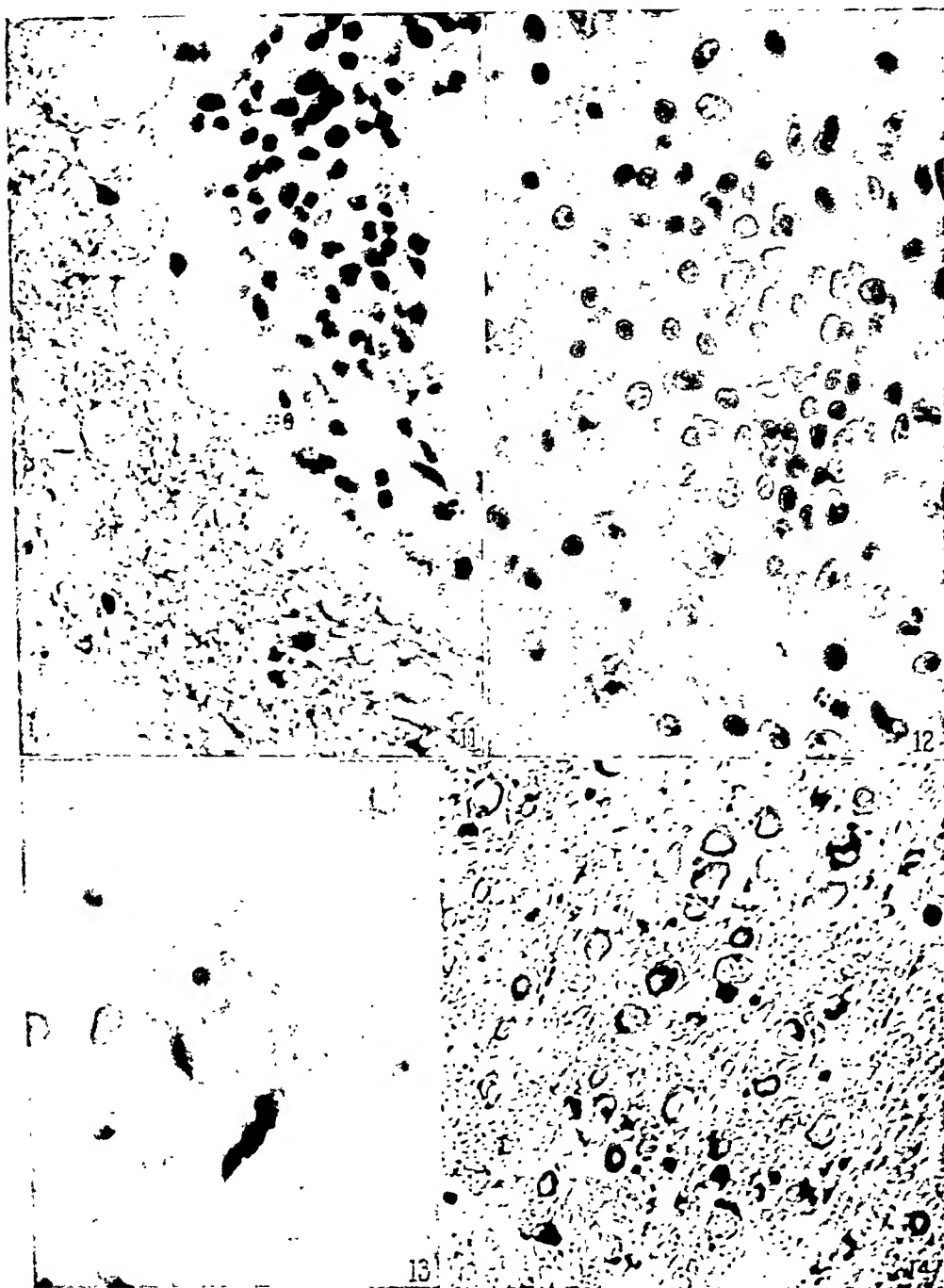
FIG. 14. Spinal cord. Degeneration of myelin-sheaths of nerve fibers in cord. Marchi's method. Swine 34. $\times 310$.



(Seiffert: Histological studies on hereditary D)



(Goldschmidt-Harris) and (Harris-Goldschmidt) (Harris-Goldschmidt)



Our failure to find a satisfactory explanation of the hemorrhages in the histology of the small vessels lead to a search for another method of approach. The purpose of the following experiment was to determine whether the resistance of the vessels to rupture is decreased in experimental scurvy, and, if so, what relation this change bears to the course of the disease.

Method

Scurvy was produced in young healthy guinea pigs weighing from 250 to 350 gm. by feeding the following diet which is deficient only in vitamin C.

	<i>per cent</i>
Baked skim milk powder (baked in shallow pan for 2 hours at 110°C.).....	30
Butter fat (butter melted and filtered at 45-50°C.).....	10
Rolled oats and bran (equal parts by volume).....	58
Sodium chloride.....	1
Cod liver oil.....	1

During the course of the disease (which, under these conditions, is approximately 3 weeks) the fragility of the skin vessels was tested by determining their resistance to negative pressure. The skin of the belly was used because other skin surfaces overlie bony structures which greatly interfere with the application of the suction cup when the animal is emaciated, as in late scurvy. The hair was removed with a barber's clipper and the skin greased with petrolatum.

The apparatus used consisted of a glass tube of 1 cm. inner diameter connected with a mercury manometer and a small hand pump. We found it advisable to use a tube with edge turned out at right angles for a breadth of 0.75 cm. to avoid constriction and afford good contact. A partial vacuum was created and maintained for 1 minute and the minimal negative pressure producing definite petechiae was taken as an expression of the vessel resistance. The 1 minute period was arbitrarily chosen. A similar apparatus was used by Hecht (9) in 1907 in distinguishing between certain erythematas and has lately been modified by Zilva-Mello (10).

A group of four animals completely deprived of vitamin C was tested every 3rd day throughout the course of the disease. On the 19th day of the diet they were given 2 cc. of orange juice by mouth from a pipette and greens *ad lib.* for the following 24 hours. During this latter period the pigs were tested every 6 hours. At the end of the period they were given 8 cc. of orange juice, and, after 6 hours, tested again.

A second group of four animals was similarly dieted but was tested every 2nd day for 2 weeks and was then left undisturbed for 10 days. On the 23rd day of the diet they were tested once to determine the degree of reaction present and then given antiscorbutics. Two animals received 12 cc. of tomato juice by mouth

A CRITERION OF HEMORRHAGIC DIATHESIS IN EXPERIMENTAL SCURVY*

By GILBERT DALLDÖRF, M.D.

(From Grasslands Hospital Laboratory, Valkhalla)

(Received for publication, October 10, 1930)

The purpose of this report is to describe the results of a simple experiment designed to investigate the hemorrhagic diathesis in scurvy.

The hemorrhagic diathesis is perhaps the most striking clinical feature of scurvy. The anatomic study of the disease, however, has centered chiefly about the changes in the skeletal system, and, lately, the teeth. This is due, in part, at least, to the absence of definite or characteristic lesions in the vessels.

The pathologic features of the terminal vessels have been described as thickening or proliferation of the cells of the intima and, by some, adventitia as well. Several pathologists consider them to be inflammatory (1, 2). According to this view of the hemorrhagic diathesis the vitamin deficiency is responsible for a nutritive state in which organisms normally non-pathogenic incite inflammatory changes. Meyer and McCormick have characterised the vessel lesions as "autolysis of small arteries (3)." Höjer (4) considers the effect of the deficiency to be an atrophy of certain types of cells and his hypothesis suggests that the vessel lesion is likewise primarily an atrophy.

Hess (7) has shown that the Rumpel-Leede phenomenon is positive in scurvy and that the tendency of the skin capillaries to rupture during stasis is responsive to treatment.

Aschoff and Koch (5) demonstrated that the skeletal lesion was a disturbance in the intercellular substances and reasoned that a like disturbance probably was present in the smaller vessels and that rupture was the result of deficient or inferior cement substances. This opinion was given support by Wolbach and Howe (6) who showed that in scorbutic animals the healing of soft tissues occurred without the formation of new capillaries though no impairment of the proliferation of endothelial cells was present.

* This work was done during the course of a visit at the Pathologisches Institut, Freiburg (Br.), Germany. The author wishes to thank the director, Geheimrat Professor L. Aschoff, for his hospitality and help and the Research Department of the United Fruit Company for financial assistance.

injected and even cyanotic before the minute occupied by the test elapsed. An area which gave a positive reading usually remained pale, the bright, cherry-red hemorrhages appearing a few seconds after the application of the suction and often increasing slowly in size during the minute interval. However, occasional areas could not be satisfactorily interpreted while under the cup. Hence, readings were always made after the cup was removed from the skin. In very faint reactions correct interpretations could be arrived at most easily by lifting the skin and looking at it by transmitted light.

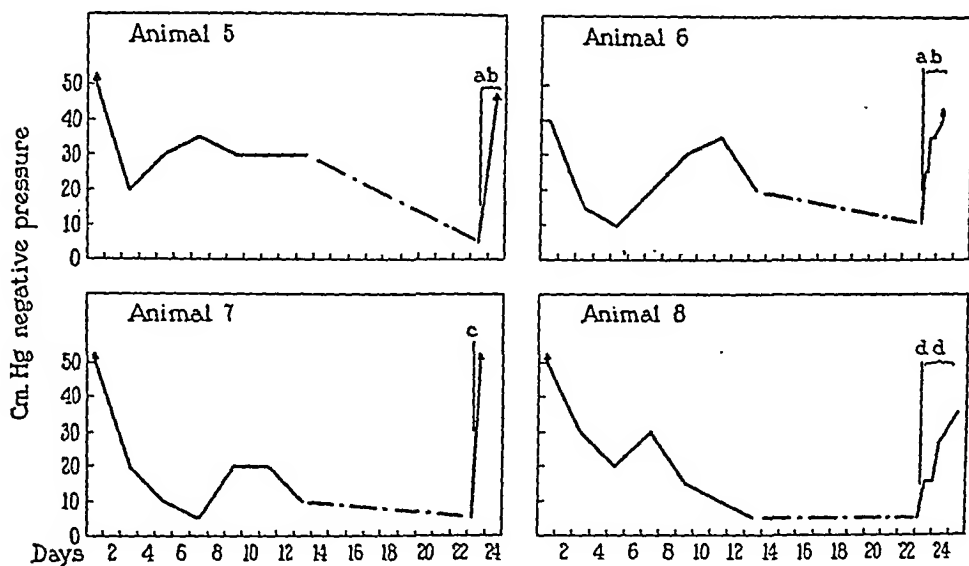


FIG. 2. Time of appearance of induced petechial hemorrhages in the skin of guinea pigs on vitamin C free diet. Second group. (a) given 12 cc. tomato juice by mouth by pipette. (b) tomato *ad lib.* at will. (c) 4 cc. banana extract intra-peritoneally. (d) banana by mouth *ad lib.* (↑) negative reading at pressure indicated. Broken line indicates interval during which no tests were made.

The degree of the reaction varied from a single fine petechia to numerous hemorrhages so large and close together as to appear to be confluent. This extensive reaction looked very much like a strawberry. The severity of the reaction in a series of readings made at one testing was roughly in proportion to the amount of negative pressure applied but since the evaluation of the degree of hemorrhage could only be approximate and it was influenced by other factors (see

(forced feeding) during the first 3 hours and tomato *ad lib.* thereafter. A third animal was given banana *ad lib.*, and a fourth received an intraperitoneal injection of an extract of banana in Locke's solution which had been purified by centrifugalization and filtration. Skin samples of areas in which petechiae had been produced were removed from two of the animals before the administration of antiscorbutics and from the same animals after the normal reaction of the vessels had been restored by the antiscorbutics. Three of the animals were tested every hour for 6 hours during the period of forced feeding, and final tests were made on all four after 24 hours. All of the animals were weighed each day they were tested.

Four other animals were tested which were part of a series in another experi-

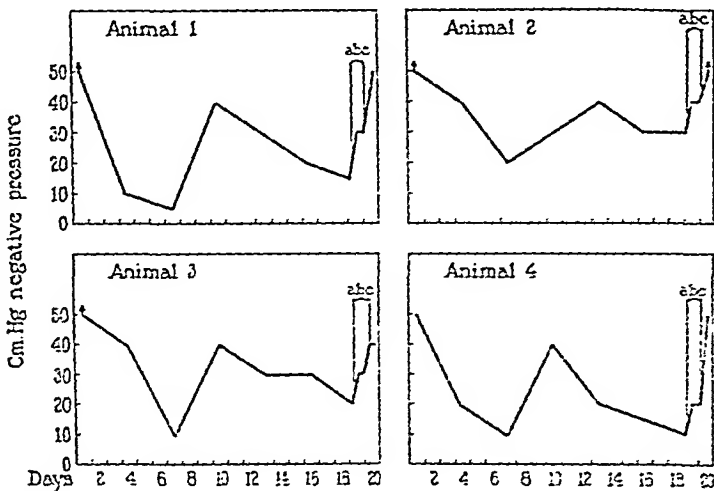


FIG. 1. Time of appearance of induced petechial hemorrhages in the skin of guinea pigs on vitamin C free diet. First group. (a) given 2 cc. of orange juice by mouth by pipette. (b) greens *ad lib.* (c) 8 cc. orange juice by mouth by pipette. (†) a negative reading at the pressure indicated.

ment and which had received the same basal diet for 6 weeks but with the addition of graded amounts of banana.

Description of the Reaction

The reading of the typical reaction to the test offered little difficulty. The result of the test was commonly indicated by the appearance of the skin dome as seen through the glass cup immediately after applying the suction. An area which reacted negatively usually became deeply

An increased liability to hemorrhage became apparent early in every case. At the end of 4 days the amount of negative pressure necessary to produce petechiae averaged 25 cm. less in one group of animals and 22.5 cm. in the other. In the tests made after 2 days of deficiency there was an increased tendency to hemorrhage in each case. The average reduction of pressure was approximately 26 cm. mercury. This downward trend occupied an average period of 6 days. Increased resistance then set in for an average period of 4 days and was followed in turn by a period of continued decline in resistance. The greatest resistance during the period of transient recovery was never equal to the normal.

In those animals kept 23 days on the scorbutic diet, hemorrhages were produced on the last test day at an average pressure of 6 cm. mercury.

Antiscorbutics were administered to all of the animals and in each case their administration was followed by prompt and definite increase in the resistance of the vessels. The greatest and most rapid improvement was found in an animal in which an aqueous extract of banana was injected intraperitoneally. This animal developed normal resistance within 3 hours of the administration of the antiscorbutic substance. In feeding antiscorbutics, more rapid recovery occurred when the protective substances were forced and in such cases the effects were noted after 6 hours. The degree of recovery, in the limited number of tests made, was proportional to the amount of antiscorbutic given. Animals of the first series showed slight but prompt recovery after 2 cc. of orange juice but ceased to improve further until given an added dose of 8 cc. of orange juice. The resistance then promptly increased again.

In four animals which had been receiving basal diet plus daily graded doses of banana over a period of 6 weeks the readings were made at irregular intervals. The average resistance in the animals receiving 3 gm. daily of banana was 10 cm. mercury, the average in the animal receiving 6 gm. was 23 cm. and the average for two animals receiving 9 gm. was 32 cm.

The skin samples removed were sectioned and stained with Giemsa solution. No differences in the structure of the terminal vessels were noted between those samples removed before treatment and those removed after recovery.

further on), the reading of most significance was held to be that of the minimal negative pressure sufficient to produce any hemorrhage whatsoever. When only one pin-point hemorrhage appeared the reading was called plus: minus and a second test made. If one or more hemorrhages appeared in the second reading the result was called positive, if none, negative. These extremely faint reactions were uncommon. The large majority of tests were frankly positive or negative.

Limitations of the Test

The most unsatisfactory factor in the experiment was the inability to test as often as desired because of the limited area of skin available, since an injured area cannot be tested again for several days. This affected both the frequency of the tests and the number of determinations made at any time.

The chief difficulty in reading positive tests arose in late scurvy when spontaneous skin hemorrhages occur. Fortunately, upon careful observation, the bright red of the new hemorrhages was easily distinguishable from the duller color of the spontaneous petechiae.

Contradictory readings at a single testing occasionally occur in the same animal in different areas submitted to the same negative pressure. This state of affairs is highly erratic in its appearance. An inconsistency appearing in an animal on one day might be found in quite different skin areas or disappear altogether the following day. The tendency bore no relation to the course of the disease.

Excitement of the animal usually affected the degree of reaction but seemed to have slight effect on the pressure at which a positive reading was obtained. This phenomenon is probably due to an increased blood pressure either acting directly to increase the tension on the vessel walls or simply causing engorgement which results in larger hemorrhages when rupture does occur.

Subcutaneous hematomas occasionally occurred in one of our animals and were associated with absence of skin petechiae in the area over the hematomas. This tendency increased with the progress of the disease.

RESULTS

The detailed results are presented in the graphs representing the behavior of the individual animals. (Figs. 1 and 2.)

theory that the underlying mechanism in scurvy is a chemical alteration of intercellular substances since it is difficult to suppose that cellular changes can occur so rapidly and since no discernible alteration in vessel structure occurred during recovery. The results of the experiment further suggest that the vitamin probably acts directly. The conception of Wolbach and Howe that the reaction during healing is analogous to the setting of a gel is in accord with our results. The theory that the vessel lesion is inflammatory in nature appears untenable.

The effect of the peritoneal injection of banana extract indicates the action to be independent of the gastrointestinal tract. This was shown by Hess and Unger when they cured scurvy with intravenous administration of antiscorbutic (12).

If the vessels of human cases are as responsive to treatment as those of the guinea pig, the vessel resistance test should have clinical value in the diagnosis of latent scurvy in which the hemorrhagic diathesis is the only sign of the disease.

CONCLUSIONS

1. The degree of scorbutic change in the vessels of animals with experimental scurvy can be roughly measured by establishing the amount of negative pressure required to produce petechial hemorrhages in the skin.

2. The test shows that the hemorrhagic diathesis in experimental scurvy develops earlier than any other known sign of the disease and that it persists in some degree throughout.

3. The response of the blood vessels to the administration of antiscorbutic substances is extremely rapid as shown by the test, but it varies with the amount of antiscorbutic given and its method of administration.

4. The changes in the resistance of the vessels follows a curve which rises towards recovery during the end of the 1st week on a scorbutic diet, reaches a peak in the 2nd week and then falls steadily during the remainder of the course of the disease. This indicates that the course of the disease is not constant and progressive.

5. The test may have clinical value in the diagnosis of scurvy.

DISCUSSION

The promptness with which changes in the vessels occur in the early stages of experimental scurvy is in accord with other features of the disease. We have repeatedly been able (10) to recognise lesions in the roots of incisor teeth after 5 days of deprivation of vitamin C. The rapidity of recovery on the administration of antiscorbutic substances is in equal agreement with the histologic changes. Wolbach and Howe (6), for example, reported definite evidence of recovery in the incisor roots within 24 hours after administering orange juice. The greater celerity of the vessel changes may be due to the nature of the test which is probably more searching than microscopic examination.

Previous observation on scorbutic animals had lead us to assume that the disease progressed regularly. The appearance of a transient period of recovery in the damage to the vessels indicates that this may not be the case. This new observation has a parallel in a well recognised phenomenon of scurvy in guinea pigs. It has long been noted that, on complete deprivation of vitamin C, the weight curve uniformly sank rapidly and then, after a few days, rose slowly to a peak greater than the original weight. Emaciation then developed rapidly until death. The transient recovery peak in the graph of vessel resistance occurs simultaneously with this peak of increased weight. We suspect that these phenomena, as yet without explanation, are due to similar causes. It is even probable that other features of the disease accord with this cycle.

The reaction might be a form of compensation due possibly to delayed mobilization of stored vitamin within the body. An observation of a possibly related phenomenon was made in Germany during the war years (11). It was observed that well nourished subjects suddenly transferred to districts in which the ration was extremely poor exhibited higher morbidity and mortality rates than the permanent population of the districts. Soldiers transferred to civil communities in which the diet was almost exclusively vegetable suffered greatly from disease and the death rate from infectious diseases was unduly high. The civil population which had gradually become reduced to the poor fare maintained a relatively great resistance to infection.

The rapidity of the change in the vessel resistance strengthens the

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units for eight Type II horses of the New York City Board of Health (Research Laboratories in Otisville, New York). The precipitin index was determined as previously described from the average of several combinations of antigen and antibody dilutions yielding a perceptible specific precipitation. The mouse protective units in the present as in our previous experiments were determined by the testing method of

TABLE I
Comparison of Precipitins and Protective Antibodies in Type II Sera

No. of horse	Precipitin index	Protective units	Ratio	Duration of treatment in months
1	768	100	7.7	12
2	384	80	4.8	21
3	384+	80	4.8	27
	448+	800	<1.0(?)	29
4	267+ } 224+ } 192+ }	80	3.3 } 2.8 } 2.4 }	27
5	362	200	1.8	27
	874+	800	1.1	29
6	768	400	1.9	29
7	320	100	3.2	42
	576	400	1.4	44
8	184	{ 100 80	1.8 2.3	42 44
Average of last five horses.....			2.1	

+ indicates prozone in precipitin test.

the Research Laboratories of the Department of Health of New York City.

The figures in Table I show (1) that a similar parallelism holds for Type II as for the other types, (2) that the average ratio of about 2 is almost half that of Type I. In other words, in Type II only half the precipitin activity of that in Type I is associated with an equal amount

THE PRECIPITIN REACTION OF ANTIPNEUMOCOCCUS SERA

III. THE RATIO OF PRECIPITIN TO PROTECTIVE ANTIBODY IN TYPE II

By HARRY SOBOTKA, Ph.D., AND MAE FRIEDLANDER, Ph.D.

(From the Lucius N. Littauer Pneumonia Research Laboratory, Department of Bacteriology, University and Bellevue Hospital Medical College, New York University, New York)

(Received for publication, November 6, 1930)

In the first paper of this series (1) it was stated that the precipitin index "offers a method for the standardization of pneumonia antibody." In a second paper (2) we concluded that "the ratio precipitin index/protective units in monovalent sera was found to lie between 2.8 and 4.8 for Type I and to be about ten times greater for Type III." The constancy of these quotients is limited by the lack of accuracy in the determination of the denominator *viz.* the amount of protective units as estimated by the uncertain mouse test.

The existence of a parallelism of the precipitin with the protective antibody in antipneumococcus sera (2) was corroborated by Heidelberger, Sia and Kendall (3) who analysed the maximum specifically precipitable protein in their attempts at a quantitative interpretation and explanation of precipitin phenomena and noticed "a definite parallel between maximum specific precipitation in Type I antipneumococcus sera and mouse protection."

The estimation of antipneumococcus antibody by precipitin tests has recently been facilitated by the rapid and convenient method proposed by Zozaya, Boyer and Clark (4). As the data of the authors mentioned are confined to Type I, and as our previous communication dealt with sera against Type I and III* only, it seemed desirable to record data on Type II precipitin and protection tests carried out during 1927-1928. Table I gives the precipitin indices and protective

* Erroneously quoted as I and II by (3).

of protective action. Types II, I and III sera exhibit increasing precipitin activity per protective titer in the order named, perhaps due to the differences in the equivalent weight of their soluble specific carbohydrates. (3) The previous observation was repeated that the precipitins reach higher titers at a faster rate than the protective antibodies during the immunizing treatment. The subsequent increase in protective activity is manifested by the drop of the precipitin/protection ratio.

With due allowance for the irregularities caused by this time factor, precipitin methods will provide a practical means for the comparison of antisera. However, some of our concentrated preparations exhibited a higher relative precipitin content than original sera (2). Sabin (5), on the other hand, was able to prepare type-specific antibody concentrates, almost or entirely free from precipitin action towards soluble specific substance. Also, Freedlander (6) produced type-specific antipneumococcus serum of high protective titer devoid of precipitins. It should be emphasized, therefore, that the precipitins do not parallel the content of protective antibodies at all in antibody preparations other than unconcentrated horse sera.

SUMMARY

The ratio precipitin/protective antibody is given for several fresh antipneumococcus horse sera (Type II).

The application of the precipitin test here dealt with and that of similar ones, based on the conception of a parallelism between precipitin and protective antibody, is limited to unrefined horse sera.

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TABLE I
Experimental Animals

Animal No.	Weight of animal kg.	Functional studies before intoxication			Dose of mercuric chloride mg. per kg.	Functional studies after intoxication				Left kidney—intravital					Right kidney—supravital				
		P.S.P. per cent	Average 24 hr. urine output	Blood urea mg./100 cc.		P.S.P.	Urine output 24 hrs. before death	Blood urea mg./100 cc.	Weight cortex gm.	Cortex counted gm.	Glomeruli counted	Glomeruli per gm.	Stained glomeruli in kidney	Weight cortex gm.	Cortex counted gm.	Glomeruli counted	Glomeruli per gm.	Total glomeruli in kidney	Open glomeruli per cent
51-52	1.5	—	—	—	10	—	0	—	4.06	0.52	7,379	14,190	57,611	4.25	0.53	16,027	30,239	128,516	44.8
55-56	1.8	—	—	—	10	—	0	—	3.56	0.53	10,683	20,157	71,759	3.96	0.59	15,363	26,039	103,114	69.5
57-58	2.1	—	—	—	20	—	0	—	2.54	0.29	5,994	20,669	52,499	2.22	0.45	16,333	36,293	80,570	65.1
113-114	2.1	70	60	20.3	20	0	0	170.8	4.31	0.50	3,119	6,238	26,886	4.89	0.52	14,255	27,413	134,049	20.0
115-116	2.0	60	130	19.3	20	0	0	135.2	4.64	0.53	9,208	17,373	80,611	4.98	0.53	13,953	26,326	131,103	61.5
117-118	2.0	80	110	17.5	20	0	0	147.4	4.35	0.52	4,119	7,921	34,456	4.81	0.50	12,009	24,003	115,454	29.8
67-68	1.8	80	140	—	20	0	11	—	4.08	0.49	10,056	20,522	83,852	3.43	0.42	18,529	44,117	151,321	55.4
111-112	2.0	75	94	23.2	20	0	15	67.4	3.99	0.50	7,500	15,000	59,850	4.62	0.53	14,104	26,611	122,943	48.7
109-110	2.2	75	120	12.9	20	10	15	64.0	5.45	0.53	8,197	15,446	84,181	5.18	0.54	13,042	24,152	125,107	67.3
69-70	1.9	80	100	—	10-48 hrs. 20	0	24	—	1.55	0.28	6,516	23,271	36,070	2.74	0.31	7,536	24,310	66,609	54.1
107-108	2.0	50	115	26.9	7-48 hrs. 7-72 hrs. 10	20	70	53.5	6.11	0.52	6,778	13,145	80,316	5.23	0.52	10,488	20,269	106,007	75.8
85-86	2.0	80	88	—	10	50	109	—	3.86	0.41	7,482	18,249	70,441	3.61	0.44	9,352	21,246	76,698	91.8
105-106	2.1	80	90	16.5	10-48 hrs. 10-72 hrs. 10	60	110	47.2	4.61	0.51	7,102	13,925	64,319	4.27	0.62	15,088	24,335	103,910	61.8

THE NUMBER OF OPEN GLOMERULI IN ACUTE
MERCURIC CHLORIDE NEPHROSIS¹BY ROBERT A. MOORE,² M.D., AND LOUIS M. HELLMAN, Ph.B.*(From the Institute of Pathology, Western Reserve University, Cleveland)**(Received for publication, November 6, 1930)*

The hypothesis that the oliguria and anuria associated with acute nephroses are due to a decreased cortical circulation, has been accepted for many years and figures in many text books. Experimental investigations to test the hypothesis have been of four types: first, measurement of renal blood flow; second, determination of the vasomotor response of the renal vessels; third, supravital measurement of the perfusion rate; and fourth, direct observation of the formation of glomerular urine. Support of the hypothesis from the clinical side is largely derived from the results of decapsulation. The literature of these observations has been recently reviewed by Hayman (1). The elaboration of an intravital method for the determination of open glomeruli by Hayman and Starr (2) offers another mode of investigation of this problem. The results of an investigation with this procedure are reported herewith.

Method

The method employed is essentially that used by Hayman and Starr (2) and Moore and Lukianoff (3). It consists of the determination of the total number of glomeruli in the right kidney by the method of Nelson (4) and the enumeration of the open glomeruli in the left kidney after intravital staining with Janus green B. On the assumption that the two kidneys contain an equal number of glomeruli, division of the latter by the former gives the per cent of open glomeruli.

Twenty-one rabbits were used, 13 experimental and 8 control. Few controls have been employed because the controls of Moore and Lukianoff (3) are appli-

¹ A part of this investigation was carried on in the Osborn Zoological Laboratory, Yale University, through the courtesy of Professor R. G. Harrison and Dr. J. S. Nichols.

² Hanna Research Fellow in Pathology.

of 63.8 per cent, which agrees well with the results of Hayman and Starr (2) and Moore and Lukianoff (3). In the experimental animals, the variation is from 20 per cent to 91 per cent, with an average of 57.3 per cent. Noting the spread of variability, the general conclusion must be, that an acute nephrosis, induced by mercuric chloride, does not influence the number of open glomeruli in the kidney. It is of interest to note that the fewest open glomeruli were observed in two oliguric animals and the most in a kaluric or polyuric animal. Since normal animals occasionally give figures within this range and since other experimental animals of this series with similar urinary output gave figures well within the normal range, it is impossible to attach significance to these three observations.

These observations make it doubtful that any success attendant upon surgical decapsulation directly depends upon alterations of cortical circulation. It is possible that other factors than the mechanical are responsible for the beneficial effects of this procedure. An investigation of this problem by the procedure used in the present study has been undertaken in this laboratory.

The results lend support to the theory of Richards (7), that the anuria of mercurial nephrosis is due to an inability of swollen and necrotic tubular epithelium to prevent a resorption of tubular urine.

SUMMARY

Acute mercurial nephrosis in the rabbit is not associated with a decrease of glomerular circulation.

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cable to this experiment. All animals were kept in metabolism cages and given 37.5 cc. of water per kilogram by stomach tube morning and evening throughout the experimental period. The normal urinary output was an average of two 24 hour periods previous to injection of the mercuric chloride. The phenolsulfonurea was determined by the urease aeration method given by Myers (5). The mercuric chloride was given hypodermatically into the tissues of the back. In three instances the injection was repeated 48 and 72 hours after the first injection. The animals were injected with Janus green B from 48 to 96 hours after intoxication, selected so that anuric, oliguric and kaluric³ animals were observed. A

TABLE II
Control Animals

Control Animals

Animal No.	Weight	Left kidney—intravital					Right kidney—supravital					Open glomeruli
		Weight cortex	Cortex counted	Glomeruli counted	Glomeruli per gm.	Stained glomeruli in kidney	Weight cortex	Cortex counted	Glomeruli counted	Glomeruli per gm.	Total glomeruli in kidney	
		kg.	gm.	gm.			gm.	gm.				
41-42	2.1	4.83	0.64	10,976	17,103	82,607						
43-44	2.5	4.23	0.55	12,463	22,660	95,852	5.29	0.73	12,809	17,546	91,941	89.8
47-48	2.1	4.38	0.58	8,243	14,212	62,248	5.89	0.64	12,307	19,233	113,282	84.6
49-50	1.8	4.40	0.54	8,984	16,637	73,203	4.24	0.56	11,960	21,357	90,553	68.7
99-100	2.0	5.59	0.68	5,622	8,268	46,218	4.40	0.62	19,394	31,280	137,632	53.2
101-102	2.1	4.13	0.55	7,281	13,238	54,673	4.74	0.67	9,003	13,437	66,379	69.6
M-1	2.2	4.38	0.52	7,194	13,835	60,597	4.18	0.53	14,175	26,745	111,794	48.9
M-2	2.0	3.96	0.48	8,126	16,929	67,039	4.56	0.51	15,281	29,933	136,494	44.3
							3.84	0.53	17,834	33,659	129,251	51.8

complete autopsy was performed
of the kidneys

complete autopsy was performed on all. Animals with non-experimental disease of the kidneys or other organs were discarded. Gross examination of the kidneys established the existence of a severe nephrosis in all the experimental animals.

The intravital injections were performed after section of the lumbar spine, as described by Ecker (6). The glomerular counts were made in the manner described by Moore and Lukianoff (3).

RESULTS

The results are given in Tables I and II. The open glomeruli in the control animals vary from 49 per cent to 89 per cent, with an average

³ The term, kaluric, has been devised to signify, as its derivation would indicate, normal urinary output.

bile, and pus from the left wrist by Dr. J. H. Bauer who identified them as belonging to the *supeistifer* group.

Case 2.—Li. Chinese boy of 6. Entered the hospital with a history of fever of 2 days duration. On admission his temperature was 40°; it gradually fell to normal 8 days later. The spleen was palpable and there was a slight jaundice. The leucocyte count was 5,150. Blood taken on the day of admission yielded a pure culture of a paratyphoid bacillus. 2 days later the blood culture was negative. The stools examined on three different occasions were negative for non-lactose-fermenting organisms and the urine was negative 4 days after admission. The serum taken 4, 11, and 23 days after admission failed to agglutinate typhoid, paratyphoid A and B, and the organism isolated from his blood. On the last two occasions, however, the hog cholera bacillus was agglutinated, in the first instance in a dilution of 1:40, and in the second 1:80. Patient was discharged from the hospital in good condition 23 days after admission.

Case 3.—Pao. Male, age 24. Entered hospital with large black gangrenous areas over the posterior aspect of both thighs, the result of a beating administered 3 days previously. The past history had no bearing on the case. His temperature on entrance was 39° and remained around this figure during his 11 days stay in the hospital. Cultures from the gangrenous areas showed hemolytic streptococci, staphylococci, and *Bacillus proteus*. 10 days after admission, the day before his death, blood was taken for culture and after defibrination, 1 cc. was transferred to each of several Petri dishes and poured plates made. From these plates, as well as from the bouillon inoculated at the same time, pure cultures of paratyphoid bacilli were obtained. It was estimated that the blood contained 15 organisms per cubic centimeter. The patient died the day after the blood culture had been made and autopsy permission was not obtained.

Case 4.—Ying. Chinese male, age 19. Entered the hospital because of a moderate distension of the abdomen and a feeling of weakness that had progressed. He stated that about a month before entrance he had had dysentery, passing bloody mucoid stools, and that some 10 days following this he had had a febrile attack which lasted 4 or 5 days. Later there was frequent urination, and it was stated that the urine was small in amount and dark in color. 2 weeks before entrance fresh blood was noticed in the urine. On entrance his face was puffy, there was edema of the legs and feet, the liver and spleen were palpable, and the heart was enlarged. He had a marked anemia, his blood cells numbering 1,864,000, with a hemoglobin content of 45 per cent. There were a few pus cells in the urine but blood was never noted. The day after admission the urine was cultured and a paratyphoid organism isolated. Subsequent cultures on four different occasions were negative, and four different blood cultures were also negative. Paratyphoid organisms were not found in 11 different stools examined. 5 days after admission his serum agglutinated paratyphoid C (Hirschfeld) in a dilution of 1:320, and 23 and 40 days later in a dilution of 1:640. The organism obtained from the urine was agglutinated in a dilution of 1:640, 20 days after admission. He was discharged in good condition about 2 months after admission.

STUDIES ON PARATYPHOID C BACILLI ISOLATED IN CHINA

By CARL TENBROECK, M.D., C. P. LI, M.D., AND H. YÜ, M.D.

(From the Department of Pathology, Peiping (Peking) Union Medical College, Peiping, China, and the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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Infections with paratyphoid bacilli are frequent in Peiping but during the course of 7 years, ending in 1927, organisms belonging to the supestifer or paratyphoid C group were detected only five times. Since these organisms are so closely related to a swine pathogen that is widely distributed and since infections with them are apparently becoming more frequent, it seems desirable for us to record briefly the history of the cases and our studies of the cultures.

HISTORY OF PATIENTS

The history of these cases was abstracted from the records of the Peiping (Peking) Union Medical College.

Case 1.—Liu. Entered the hospital in 1922, with a history that 20 days before a temperature had developed together with chills and headache, and that this had been followed by jaundice. Some days later there was swelling and pain in several of the joints, especially those of the left wrist and hand. He admitted having had gonorrhea 10 years and 6 months before entrance. An examination showed a purulent discharge from the urethra which contained Gram-negative intracellular diplococci. His temperature was 38° on entrance and did not go above this figure. 7 days after entrance he developed a petechial rash, and from a blood culture a pure growth of paratyphoids belonging to the supestifer group was obtained. He died the following day and the anatomical diagnosis made from the autopsy was: Chronic gonococcal urethritis, prostatitis, and seminal vesiculitis with abscess formation; acute vegetative mitral endocarditis; acute splenic tumor; acute cholecystitis; and nodular cirrhosis of the liver. Gonococci were found in films from the prostate and in pus from the left wrist, and were grown from the spleen. In addition, paratyphoid bacilli were grown from the spleen, heart blood,

Absorption tests were at first confusing, and it was only after we had adopted the method of Andrewes (1) and produced specific and non-specific sera that we could come to any conclusions regarding the true position of these cultures.

Bouillon cultures made from isolated and tested colonies and killed by the addition of 0.15 per cent formalin were used for the production of the antisera.

TABLE I

Group	Culture	Action on		
		Rhamnose	Arabinose	Dulcitol
I	Ying Chao Paratyphoid C (Hirschfeld)	Acid only at first. Later small amount of gas	Acid and gas in 24 hours	Acid and gas in 24 hours
II	Liu Pao Li	Acid and gas in 24 hours	No acid or gas	Acid and small amount of gas after 5 days
Hog cholera bacillus	HGXVI	"	" " " "	No acid or gas

TABLE II

Serum	Agglutinates organisms of		
	Group I	Group II	Hog cholera
Group I	To titer limit	In low dilutions only	To titer limit
" II	In low dilutions only	To titer limit	" " "
Hog cholera	To titer limit	" " "	" " "

As a rule three intravenous injections into rabbits at intervals of a week in doses of 0.1, 0.25, and 0.5 cc. produced a satisfactory agglutinating serum. For the agglutination tests as well as the absorptions living cultures were used. The various forms were kept dried on papers according to the method of Brown (2) and when needed flasks of bouillon for antigens or Blake bottles for absorptions were inoculated. This method proved to be a very satisfactory and labor-saving one.

It was soon evident after these sera had been prepared that the cultures of Group I contained both specific and non-specific forms, or as

Case 5.—Chao. Chinese woman, age 24. Entered another hospital with evidence of sepsis and with a history of abortion induced 4 days previously. She had pain and extreme tenderness in the right hypochondrium. There was moderate jaundice. 12 days after entrance her temperature began to rise and had the characters of a typhoid-like disease. There is no note of the presence of rose spots or of enlargement of the spleen. Towards the end of this febrile attack blood was taken for culture and a paratyphoid organism isolated. This same organism was found in the urine 31 and 33 days after admission, and in one stool culture 35 days after admission. 33 days after admission the patient's serum agglutinated the organism isolated from the blood in a dilution of 1:640. There was no agglutination of *B. typhosus*, or of paratyphoid A or B. 40 days after admission the serum agglutinated the patient's own organism and the hog cholera bacillus in a dilution of 1:2,560.

The organisms isolated from these five cases were compared with our stock cultures of paratyphoid A and B and with paratyphoid C of Hirschfeld as well as with the hog cholera bacillus.

Cultural Tests

All were Gram-negative, actively motile rods, that formed acid and gas promptly from dextrose, maltose, mannitol, and xylose, and failed to act on saccharose and lactose. They did not liquefy gelatin or produce indol. By their action on rhamnose, arabinose, and dulcitol they could be divided into two groups, as is shown in Table I, which also includes the hog cholera bacillus.

Cultures Ying and Chao failed to produce hydrogen sulfid soon after isolation but they later acquired this property. On the other hand, our records show that the organisms of Group II formed hydrogen sulfid immediately after isolation.

Serological Tests

All of the cultures were agglutinated in low dilutions of typhoid and paratyphoid B but failed to show clumps in paratyphoid A and *enteritidis* antisera. In anti-hog cholera bacillus serum all of the cultures were agglutinated to the titer limit, and the sera of rabbits immunized to these cultures agglutinated the hog cholera bacillus and the members of the group to which they belonged. The organisms of the other group, which had been distinguished by cultural characters, were agglutinated in low dilutions only. This is summarized in Table II.

TABLE IV

Serum used		Titer limit for immunizing culture after absorbing with										
		Specific phase of diphasic cultures			Non-specific phase of diphasic cultures			Non-specific phase of diphasic cultures			Permanently non-specific cultures	
		HCXVI	Para. C	Ying	Chao	HCXVI	Para. C	Ying	Chao	Li	Pao	Liu
Rabbit No.	Immune to	Control										
1	HCXVI	12,800	0	200	200	1,600	3,200	800	6,400	12,800	12,800	12,800
2	Para. C	12,800	200	—	—	3,200	—	—	6,400	12,800	—	—
3	Ying	12,800	0	200	0	800	6,400	1,600	6,400	12,800	—	—
4	Chao	12,800	400	0	0	800	3,200	400	800	12,800	12,800	12,800

TABLE V

Serum used		Titer limit for immunizing culture after absorbing with										
		Specific phase of diphasic cultures			Non-specific phase of diphasic cultures			Non-specific phase of diphasic cultures			Permanently non-specific cultures	
		HCXVI	Para. C	Ying	Chao	HCXVI	Para. C	Ying	Chao	Li	Pao	Liu
Rabbit No.	Immune to	Control										
5	HCXVI	6,400	0	3,200	400	1,600	0	0	0	0	0	0
6	Para. C	6,400	400	3,200	800	6,400	0	0	0	0	0	0
7	Ying	12,800	3,200	12,800	6,400	12,800	0	200	3,200	0	0	200
8	Chao	12,800	800	6,400	800	6,400	0	0	100	0	0	0
9	Pao	12,800	800	6,400	6,400	6,400	0	800	1,600	0	0	0
10	Liu	6,400	200	3,200	400	800	0	100	200	0	0	0

TABLE III

Serum used		Titer limit of agglutinating serum for											
Rabbit No.	Immune to	Phase	Specific phase of diphasic cultures			Non-specific phase of diphasic cultures						Permanently non-specific cultures	
			HICXVI	Para. C	Ying	Chao	HICXVI	Para. C	Ying	Chao	Li	Pao	Liu
1	HICXVI Para. C Ying Chao	Specific	12,000	25,600	25,600	25,600	1,600	1,600	3,200	800	1,600	1,600	3,200
2		"	12,800	12,800	25,600	25,600	1,600	800	400	1,600	800	1,600	800
3		"	12,800	12,800	25,600	25,600	1,600	800	3,200	800	800	800	3,200
4		"	6,400	6,400	6,400	12,800	800	1,600	800	1,600	1,600	800	3,200
5	HICXVI Para. C Ying Chao	Non-specific	1,600	3,200	1,600	1,600	6,400	3,200	6,400	12,800	6,400	6,400	12,800
6		"	800	800	800	1,600	1,600	12,800	12,800	12,800	12,800	12,800	12,800
7		"	3,200	6,400	3,200	6,400	12,800	12,800	12,800	12,800	12,800	12,800	12,800
8		"	800	3,200	3,200	3,200	6,400	6,400	6,400	12,800	12,800	12,800	12,800
9	Pao Liu	"	0	0	0	0	1,600	1,600	3,200	1,600	6,400	6,400	6,400
10		"	0	0	0	0	1,600	1,600	1,600	3,200	3,200	3,200	6,400

and that small amounts given subcutaneously will produce a febrile disease followed by death in from 8 to 10 days after inoculation. While members of the paratyphoid group if injected intraperitoneally or intravenously will kill rabbits, they produce little or no effect when injected subcutaneously in amounts of 0.1 cc. or less of 24 hour bouillon cultures. The hog cholera bacillus, on the other hand, even in dilutions as high as 1:1,000,000 will kill rabbits, provided the precaution is taken to test the animals before injection to see that they are not carriers of the rabbit paratyphoid organism, for it has been shown that an infection with this organism will protect against the hog cholera bacillus (4).

When these five cultures were tested for their virulence, it was found that those in Group I were not virulent for rabbits, whereas those belonging to Group II were, so far as we could tell, just as virulent as the hog cholera bacillus. 0.1 cc. of a 24 hour bouillon culture of a Group I organism injected subcutaneously produced little or no effect, while 0.000001 cc. of any of the three organisms belonging to Group II killed rabbits in less than 10 days.

DISCUSSION

The division of paratyphoid C organisms into two groups was made first by Andrewes and Neave (5) and was confirmed by White (6) and others. We have added simply the virulence of the non-specific organisms for rabbits. This we feel is of some importance for it brings them more closely to the hog cholera bacillus, as does their action on rhamnose, arabinose, and dulcitol.

We have no knowledge of the types of hog cholera bacilli found in swine in China, but White (6) has pointed out that the forms found in western Europe differ from those found in the United States, eastern Asia, and Europe, in that the majority of the former yield the non-specific form only while the majority of the latter are diphasic. Confirming Jordan and Victorson's work (7), he notes that most cultures from America failed to produce H_2S , while the European strains promptly blackened lead acetate medium.

Recently one of us isolated from swine in the United States a number of purely non-specific hog cholera bacilli. These forms appear to be identical with the organisms of Group II isolated in China as well as

White says, they were diphasic, while the cultures of Group II were permanently non-specific.

In Table III are given the results of agglutination tests with various sera. It will be seen that the specific sera of the hog cholera bacillus as well as the serum from the animal immunized to the specific phase of the organisms of Group I agglutinate the specific phase to practically the titer limit in every case; that the non-specific phase of the diphasic cultures is agglutinated in low dilutions only; and that organisms of Group II are agglutinated very slightly by these sera. It will also be seen that the sera of animals immunized to the non-specific phase of the diphasic cultures agglutinate the specific phase to a certain extent but not to the titer limit of the sera, whereas the non-specific phase and the organisms of Group II are agglutinated to practically the titer limit. Further, the sera of rabbits immunized to Group II organisms fail to agglutinate the specific phase of cultures but do agglutinate the non-specific phase as well as the organisms of Group II.

Absorption tests are given in Tables IV and V, and it should be noted that in these tables the figures are the highest dilutions of absorbed sera that agglutinate the cultures used in producing the serum. It will be seen that the specific phase of the diphasic cultures absorbs practically all the agglutinins from the specific sera, and that while the non-specific phase lowers the titer it does not completely remove them; also that the organisms of Group II have no effect on the specific sera. It is evident that the non-specific phase has some specific agglutininogen present.

In Table V are given the results of absorption tests on the sera of rabbits immunized to the non-specific phase and on the sera of rabbits immunized to the permanently non-specific organisms of Group II. It will be seen that while the specific phase tends to lower the titer of these sera, it does not by any means remove all the agglutinins; whereas, with a few exceptions, the non-specific phase and the permanently non-specific forms completely remove the agglutinins from the immunizing culture.

Virulence for Rabbits

Theobald Smith (3) in his first descriptions of the hog cholera bacillus pointed out that these organisms are extremely virulent for rabbits

the western European forms found by White. We have also had an opportunity to study the culture isolated from man by Branham, Motyca, and Devine (8) and find that in its cultural and serological reactions it is a purely non-specific form. Since reports of the isolation of this group of organisms from humans are becoming more frequent, we would suggest that studies include the pathogenicity for rabbits and the serological classification. The possible infection from swine should also be considered. In the cases we have studied it was impossible for us to establish any relationship with pigs.

What part these organisms played in the diseases of these five patients did not come within the scope of these studies. It seems probable that in the majority of these cases they were present as secondary or terminal invaders.

SUMMARY

A brief clinical history of five patients from whom paratyphoid C was isolated and a description of the cultures are given. It is pointed out that the purely non-specific forms are more nearly related to the hog cholera bacillus than are those containing both specific and non-specific forms.

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TABLE I

No.	Control group						Operated group								
	Gross body weight		Corrected body weight	Body length	Spleen weight	Spleen mg. per 100 gm. body weight	Gross body weight		Corrected body weight	Body length	Pole of spleen removed	Weight of spleen removed	Spleen weight at death	Spleen mg. per 100 gm. body weight	
	Initial	Death					Initial	Death							
10 days after operation															
1	gm. 216	gm. 280	gm. 274	mm. 213	mg. 1047	382	gm. 238	gm. 260	gm. 255	mm. 212	U	mg. 472	mg 702	275	
2	242	290	290	220	1264	436	216	254	245	220	L	469	795	324	
3	238	286	283	216	1130	399	216	250	245	204	U	427	635	259	
4	266	326	320	227	988	309	262	270	265	213	L	456	676	255	
5	229	286	281	215	1018	363	211	240	236	201	U	612	808	343	
6	214	254	251	207	1381	550	226	264	257	214	L	581	1001	390	
7	187	242	235	206	1591	677	202	234	229	207	U	683	1170	511	
8	218	265	264	211	1386	525	198	224	218	204	L	548	971	446	
9	183	198	195	201	958	491	211	231	228	202	U	580	803	353	
10	202	251	249	205	1011	406	194	222	217	208	L	540	594	274	
Mean.....						454	Mean.....						343		
20 days after operation															
11	241	288	288	221	1062	369	245	318	317	225	U	511	895	283	
12	269	306	305	228	1346	442	271	328	223	223	L	745	858	385	
13	286	342	235	226	1240	527	212	270	270	214	U	510	1158	429	
14	203	248	246	208	982	399	233	284	283	220	L	513	1004	355	
15	210	260	255	213	1526	598	230	265	263	213	U	686	855	325	
16	244	294	293	226	1504	513	220	275	273	209	L	576	696	255	
17	197	234	232	208	901	389	210	278	275	218	U	398	936	340	
18	198	246	243	222	864	356	229	252	254	210	L	354	675	266	
19	207	246	246	209	778	317	203	250	248	207	U	398	672	271	
20	221	282	280	216	766	273	192	242	237	205	L	303	726	306	
Mean.....						418	Mean.....						322		
40 days after operation															
21	232	326	317	220	1169	369	210	251	247	210	L	628	703	285	
22	178	230	221	205	973	441	182	244	237	210	U	336	653	277	
23	232	286	279	222	920	329	224	264	262	212	L	400	556	212	
24	212	256	246	210	897	364	224	250	248	212	U	440	890	359	
25	228	286	279	218	939	336	205	252	249	212	L	424	592	238	
26	230	292	285	227	1114	390	225	290	282	219	U	488	722	257	
27	226	278	272	219	926	341	212	260	251	212	L	485	500	200	
28	216	302	294	220	847	288	222	276	267	216	U	338	537	201	
29	218	300	297	222	976	328	216	281	270	213	L	364	638	236	
30	212	281	276	217	924	335	212	288	277	216	U	354	582	210	
Mean.....						352	Mean.....						248		

COMPENSATORY HYPERTROPHY OF THE SPLEEN*

By EATON M. MacKAY, M.D., AND W. SCOTT POLLAND, M.D.

*(From the Department of Medicine, Stanford University Medical School,
San Francisco)*

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Following the removal of the spleen, at least in man and the dog, there may be an hypertrophy of accessory splenic tissue situated elsewhere in the abdomen. This is often spoken of as regeneration. It is not however true regeneration. By the process of regeneration in the gross sense is meant the restoration of a tissue at the site of its removal (e.g., skin in the healing of a superficial wound) as opposed to the restoration, usually partial, of a tissue elsewhere than at the site of extirpation, a process which is referred to as compensatory hypertrophy. This does not imply that it is due solely to an hypertrophy of the individual tissue elements for the term is applied to the tissue mass as a whole. Compensatory hypertrophy when used to denote the changes which ensue in a remaining tissue following the removal of similar tissue elsewhere applies only to the gross increase in size and may be brought about by hyperplasia, hypertrophy of the individual tissue cells or both. In connection with the interest in this laboratory (1, 2, 3) in the general subject of compensatory hypertrophy observations have been made upon the occurrence and extent of compensatory hypertrophy following the removal of a part of the spleen.

Methods

Experiments were carried out with both rats and rabbits. These were essentially the same. A number of animals of similar age and sex were divided into two groups. One of these served as a control while in the other group half of the spleen was removed from each animal. The operations were performed under ether anesthesia. In the control group the spleen was exposed through a small incision in the abdominal wall. In the other group the peritoneal cavity was exposed by an incision in the upper left quadrant of the abdominal wall near the back muscles.

* This investigation was made possible by the Ella Sachs Plotz Fund.

COMPENSATORY HYPERTROPHY OF THE SPLEEN

TABLE II

TABLE II							
No.	Body weight		Weight alimentary tract	Net body weight	Pole of spleen removed	Spleen weight	Spleen weight
	Initial	Death					
Control group							
	kg.	kg.	kg.	kg.		mg.	mg. per kg.
1	2.30	2.60	0.40	2.20		1332	566
2	2.15	2.50	0.60	1.90		725	381
3	2.10	2.50	0.55	1.95		1191	611
4	2.05	2.60	0.55	2.01		1070	531
5	2.00	2.35	0.55	1.80		1634	907
6	1.95	2.05	0.40	1.65		1799	1090
7	1.91	2.45	0.70	1.75		607	347
8	1.85	2.40	0.53	1.87		1260	674
9	1.85	2.45	0.45	2.00		1782	890
10	1.81	2.20	0.47	1.73		1351	780
11	1.75	2.35	0.40	1.95		1102	565
12	1.71	2.10	0.43	1.67		897	537
13	1.70	2.20	0.34	1.86		632	340
14	1.60	2.05	0.40	1.65		770	467
15	1.45	2.05	0.35	1.70		980	576
Mean.....							617

Operated group							
1	2.20	2.55	0.58	1.98	L	794	401
2	2.05	2.45	0.60	1.85	U	955	515
3	2.05	2.60	0.70	1.90	L	684	359
4	1.95	2.50	0.57	1.93	L	306	158
5	1.90	2.20	0.55	1.65	U	682	425
6	1.90	2.22	0.48	1.81	L	459	253
7	1.80	2.35	0.45	1.90	U	645	339
8	1.80	2.20	0.45	1.75	L	540	309
9	1.70	2.05	0.28	1.78	U	373	210
10	1.70	2.05	0.33	1.73	L	520	300
11	1.70	2.20	0.40	1.80	U	479	266
12	1.70	2.40	0.43	1.98	L	751	379
13	1.50	1.85	0.35	1.50	U	634	425
14	1.45	1.85	0.25	1.60	L	603	377
Mean.....							337

The spleen was gently drawn out of the cavity and cut in two as nearly as possible in equal halves. After ligation of the blood supply by a gut ligature inserted through the mesentery and tied above that half of the mesentery corresponding to one portion of the organ this pole was removed. The peritoneum and muscles were closed with a simple gut suture and the skin brought together with suture clips. The important point at this juncture concerns just how near half of the spleen was removed at operation. In a trial series of ten rats in which the spleen was brought to the abdominal wall as in our operations and halved *in situ* purely by inspection and then the remaining pole removed and both weighed after removal of the oozed blood, it was found that the greatest deviation of the weight of any one of the half spleens from the mean weight of the two halves was less than 10 per cent. In the rabbit in which the spleen is a more irregularly shaped organ than in the rat the maximum deviation in a series of ten was slightly over 15 per cent. These differences are not much greater than the differences in weight of some of the paired organs.

Rat Experiments

Sixty albino male rats 130 days of age were divided into a control and a half splenectomized group of equal number. At 10, 20 and 40 days after operation ten rats from each group were etherized and then bled to death from the abdominal aorta, a procedure which should reduce the amount of extraneous blood in the spleen to a minimum (4). The spleens were then removed and weighed. The half spleens had the same appearance as at operation except for having increased in size. In no case was there any evidence of necrosis or scarring of the cut end.

That there was a definite compensatory hypertrophy of the half spleen remaining after bisplenectomy is evident from Table I. This amounted to 51.1, 54.0 and 41.0 per cent respectively 10, 20, and 40 days after operation. The process was therefore complete in 10 days. Rather than attach statistical meaning to the averages of such relatively small series of biological observations they have been compared graphically in Fig. 1 and from this it seems certain that there can be no question as to the results being significant.

The histological examination of the spleens showed nothing unusual. In both the control and hypertrophic organs the splenic follicles were the characteristic enlarged prominent follicles of the *Bartonella* infected rat (see discussion). The increase in splenic tissue in the organs undergoing compensatory hypertrophy was due to an increase in the pulp.

the rat and rabbit. We believe that their conditions are more or less akin to ours and that the explanation may not be as simple as this.

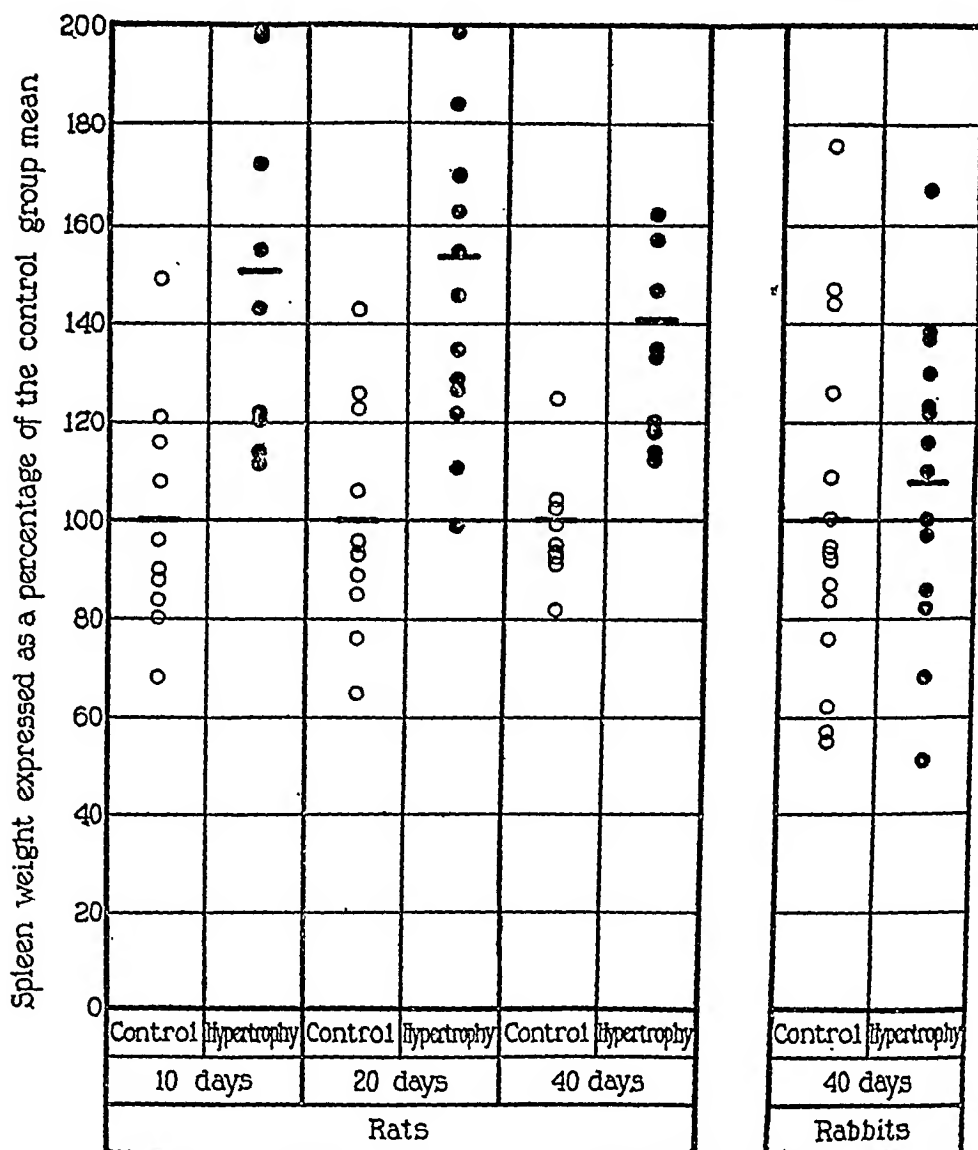


FIG. 1

Our rabbits were not infected with *Bartonella muris* and there is no reason to believe that those of Marine and Manley (7) were. Rabbits are not known to be infected naturally and only with great difficulty can young rabbits be infected experimentally and adult

Rabbit Experiment

It has been suggested (5) that the ratio of the weight of the spleen to that of the body indicates that the spleen is relatively of more significance in the rat than in some other animals and of least importance in the rabbit. It occurred to us then that compensatory hypertrophy of the spleen while it followed the removal of half the organ in the rat might not occur in the rabbit where the tissue appeared to be more or less unessential. An experiment was carried out to test this possibility.

Thirty-two young adult rabbits were divided into two groups on the basis of paired litter mates of the same sex. Half of the spleen was removed from one of these groups in the manner which has been described. One of the operated animals died soon after the operation. All of the rest including the control group were killed 40 days later and the splenic tissue removed and weighed.

The data in Table II and Fig. 1 give no evidence of compensatory hypertrophy of the remaining tissue following removal of half of the spleen. Histologically the splenic tissue of both the control and operated groups was the same.

DISCUSSION

While the adrenal cortex of the rabbit has been found (6) to undergo a considerably less degree of compensatory hypertrophy than similar tissue in the rat there is a still greater difference in their splenic tissue for while this shows no measureable compensatory hypertrophy in the rabbit there is no question as to its occurrence in the rat. One is tempted from the experiments reported here to conclude that the spleen of the rabbit is fundamentally different from the same tissue in the rat. In the rabbit, Marine and Manley (7) showed that though splenic autotransplants will grow in the adult rabbit in the absence of the spleen in many instances, these transplants are often resorbed or remain small and do not undergo hypertrophy. In the rat, however, Perla and Gottesman (8) found that splenic autotransplants will grow even in the presence of the spleen and in its absence will undergo marked hypertrophy. These investigators conclude that this indicates a difference in the function of the adult spleen of

SUMMARY

Following the removal of half of the spleen in young adult rats there is a compensatory enlargement of the remaining portion of about 100 per cent while in mature rabbits no such compensatory hypertrophy follows a similar procedure. It is uncertain whether this difference is due to a difference in the function of the spleen in the two species or to the fact that the rats were infected with *Bartonella muris* while the rabbits were not.

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rabbits rarely if at all. Our rats on the other hand were infected with this organism as shown by the fact that any member of the strain rapidly developed the characteristic anemia which *Bartonella muris* causes when the spleen is removed (9, 10). The rats used by Perla and Gottesman (8) were also without question infected with this organism. Now the latent *Bartonella muris* infection which is present in the large majority of albino rats is responsible for the enormous spleens these animals usually have. Cannon and McClelland (11) find that the infection causes the spleen to enlarge to three times its normal size. If this splenic tissue is re-duced by an autotransplant it is reasonable to assume that the trans-plant will take and that it will increase in size if the bulk of the parent tissue is still further reduced. Furthermore since the splenic tissue, due to the latent infection, is present in an amount far above normal it might be expected, when half of the organ was removed, to increase in size as a result of the presence of this abnormal stimulus. Is the differing behavior of splenic transplants in adult rabbits and rats (8) and the difference, which is of essentially the same nature, in the compensatory hypertrophy of the spleen in these two animals, due to some fundamental difference in the splenic tissue of the two species or to the fact that the rats possess a stimulus to the hyper-trophy of splenic tissue in their *Bartonella muris* infection while the rabbit does not? We are inclined to the latter view. The present data are insufficient to settle the point. It is possible that a part of the compensatory hypertrophy of the spleen of the rat is genuine in the sense that it would take place in the healthy animal. This could be determined by repeating the rat experiments described here upon the special Wistar strain (11) of uninfected rats. If it is due to the *Bartonella muris* infection one would expect young rabbits infected (8) with this organism, to show compensatory hypertrophy of the spleen. It is possible of course that very young rabbits might show compen-satory splenic hypertrophy for Marine and Manley (7) found that they differ much from the mature animal and react to splenic transplants in essentially the same manner as the mature *Bartonella muris* infected rats of Perla and Gottesman (8). It is hoped that the difference in the behavior of the spleen in the rat and the rabbit can at a later period be examined in the manner proposed.

munization purposes. For we believed that in this disease, as suggested by Spencer and Parker and by Conner for Rocky Mountain spotted fever, success in protective vaccination depends upon the use of a concentrated virus in order that relatively large doses of the killed material may be given. The diet experiments seemed to us to have supplied a method which will perhaps solve this problem in a way sufficiently practical for human test. Meanwhile, in the course of developing the diet method, we have continued experimentation on active immunization with formalinized material from the tunica lesions in guinea pigs containing fair concentrations of *Rickettsiae*. Two such experiments are detailed below, since we believe that they corroborate the earlier experience referred to in our Typhus Fever Studies I (1) and confirm the observation that active immunization with killed virus is possible in this disease.

Experiment I.—Six male guinea pigs were inoculated on June 27th, July 2nd and July 13th, respectively, with suspensions of ground tunica material containing *Rickettsiae* in 0.2 per cent formalin in salt solution. None of the guinea pigs received more than the contents of about one-third of a single tunica at each injection, in total volumes of 5, 2 and 4 cc., respectively.

In three of the animals the vaccine injections aroused no noticeable reaction. In the other three of them there was a sharp rise of temperature (twice in two of the animals, once in the other) on the day following the vaccine administration. In every case these prompt fever reactions—reaching 104°F.—came too soon to be attributed to living virus, and were not sustained, receding to normal within a day. We attribute them to the toxicity of the material injected.

On August 3rd, 31 days after the last vaccination, the six guinea pigs were inoculated with a large dose of European typhus virus, that is, they received 2.5 cc. of a mixture of defibrinated blood and brain of a European typhus animal, taken on the second day of its fever, when the temperature was 105°F. Chart 1 shows the results of these inoculations. (Vaccinated Guinea pig 6 is not charted for purposes of economy of space. It was in significance equivalent to vaccinated Guinea pig 1, showing a definite but mild typhus reaction.) The three controls charted all developed a typical and severe experimental typhus—severe in the promptness with which the temperature rose above 104°F., namely, in all cases on the 6th day, and in the length of time for which it remained between 104°F. and 106°F.—in one case for 8 days and the other two for 9 days. In the vaccinated animals, the two least successful vaccinations—namely, Guinea pig 1 and the uncharted animal—there were rises of temperature postponed until the 8th and the 9th days, respectively, lasting above 104°F. for only 4 and 5 days. Vaccinated Guinea pig 3 seemed entirely immune, and in the remaining three animals the

STUDIES ON TYPHUS FEVER

V. ACTIVE IMMUNIZATION AGAINST TYPHUS FEVER WITH FORMALINIZED VIRUS

By HANS ZINSSER, M.D., AND M. RUIZ CASTANEDA, M.D.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston)

(Received for publication, December 5, 1930)

In a preceding paper on typhus fever published by one of us with Batchelder (1) we reported on immunization experiments in which it was found that convalescent blood from typhus guinea pigs, mixed in the test tube with virus, affords protection if the convalescent blood is taken between the 1st and the 10th days after defervescence. In a limited number of animals negatively inoculated with virus-serum mixtures, reinoculation with virus alone proved them to have been immunized. Similar observations had been made by Nicolle (2) and we regarded this as an active immunization by a living virus, rather than a passive immunization by the serum. Encouraged by this, as well as by experiments of Spencer and Parker (3) and of Conner (4) with Rocky Mountain spotted fever, we attempted to immunize guinea pigs with tunica containing *Rickettsiae* ground in sand and left overnight at room temperature in a 0.2 per cent formalin salt solution. Two animals so treated, and not sustaining experimental typhus as a result of the vaccinations, proved immune when reinoculated with virus 48 days after the last vaccination. In the first vaccinations, large amounts of the formalinized virus were injected and subsequent experiments, in which material much less rich in *Rickettsiae* and in smaller amounts was used, were inconclusive. Benzol experiments elsewhere reported (5) and the diet experiments to be published (6) had as their purpose not only the investigation of the etiological significance of the *Rickettsiae* but also the search for a method of sufficiently concentrating *Rickettsia* material for im-

Experiment II.—On October 11th, five male guinea pigs were inoculated with formalinized tunica material prepared in a manner similar to that described above. The material was richer than that used in the preceding experiment, but was not yet comparable to the material now made available by the diet method with which we are working at the present time. The vaccinations were repeated intraperitoneally on October 16th and 21st, respectively. In the course of vaccination three of these animals on either one or two occasions had sharp rises of temperature to 104°F. on the day after vaccination, but dropped to normal again on the day following. On November 6th, 17 days after the last vaccination, these animals, together with two controls, received a relatively small dose of European typhus virus. The inoculation consisted of mixed defibrinated blood and brain taken from an animal in the 13th day of European typhus infection, when the temperature was 105.8°F. Chart 2 shows the results of this inoculation. Control 1 sustained a prolonged temperature curve which remained above 104°F. for 11 days—except for one temporary drop on the afternoon of the 11th day of disease—rising to 105°F. on the 17th day. Control 2 sustained a temperature reaching 105°F. on the 10th day, the temperature being maintained for 3 days, then dropping for 2 days and going up again above 104°F. for 3 more days—a condition which we would interpret as a mild but distinct typhus comparable in severity to that of vaccinated Guinea pig 1. Vaccinated Guinea pig 1 also showed a typhus fever reaction not materially milder in severity from that of Control 2. The remaining three vaccinated animals were immune.

The charts are not carried further in the tabulation because after the 20th day there were no further rises of temperature.

In this experiment, then, three out of five vaccinated guinea pigs failed to show any reaction after inoculation with a virus which produced distinct fever curves in two controls and in one of the remaining vaccinated animals, and a suggestive fever curve in another vaccinated animal.

Although there could be no reasonable question as to the fact that the controls and the vaccinated Animal 1 in these charts exhibit typical though mild typhus fever curves, to make assurance doubly sure we reinoculated all of these animals—controls and vaccinated animals—with a strong dose of Mexican typhus virus to which a normal animal reacted with typical temperature on the 5th and scrotal swelling with *Rickettsiae* on the 6th day. All of these animals remained immune, proving that the controls and the mildly reacting vaccinated guinea pig really had typhus fever and that the negative reactions in vaccinated Guinea pigs 3, 4 and 5 were due to vaccination immunity and not to accident or impotence of the virus.

evidences of infection took the form of short-lived rises of temperature which in only one of them—No. 2 on the chart—rose above 104°F ., and in No. 4 and No. 5 (which are shaded between 103° and 104° in order to call particular attention to these temperature increases) just touched 104°F . for 1 and 2 days, respectively.

It is apparent from this experiment that, by active immunization with formalinized and relatively sparse *Rickettsia* material, it has been possible to protect guinea pigs to a degree such that two out of

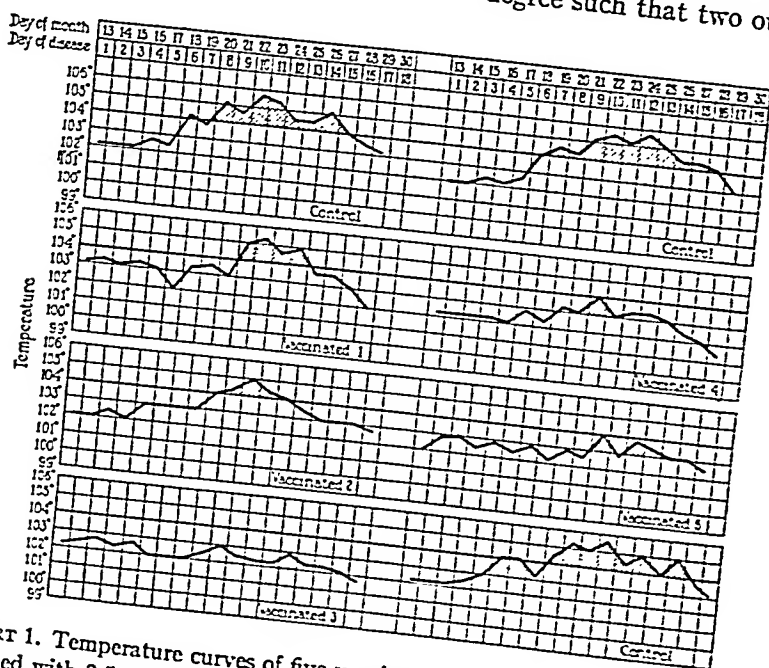


CHART 1. Temperature curves of five vaccinated and three control guinea pigs inoculated with 2.5 cc. of European typhus brain and blood, as described in Experiment I.

the six animals had a relatively moderate typhus infection, three had what might fairly be called aborted infections, and one guinea pig remained entirely immune.

This experiment, together with the preceding one reported by Batchelder and one of us, increased our belief that the immunization was a real one, and not simulated by accidental differences of the guinea pigs because, though we completely immunized in only one animal, there was an obvious modification of the disease in the direction of mildness in all the others.

the formalin solution were dead or living. For it is of the greatest theoretical and practical importance whether we actually accomplished vaccination with dead virus, or whether we have been working with an attenuated but still living organism. The latter possibility is suggested by the occasional and temporary sharp rises of temperature following vaccination. Yet we believe that it is quite unlikely that these formalinized preparations contained living *Rickettsiae*, for the following reasons. In the first place, the temperature reactions, when they did occur, which was not always, came within a day after injection, which is far too soon for a reaction to living virus. Again, in no cases were temperatures sustained, nor was there ever a swelling of the testes such as that which is invariably obtained after intraperitoneal inoculation of male guinea pigs with the living virus of the Mexican type which was used in preparing the vaccines.

In vaccination experiments which we are carrying out at the present time, and in which we are working with peritoneal exudates of diet animals in which *Rickettsiae* are present in almost cultural amounts, we find that there is distinct evidence of a powerful toxic reaction consisting of sickness and a sharp, but unsustained temperature rise after the administration of this concentrated form of vaccine.

CONCLUSIONS

We have adduced evidence that guinea pigs can be completely or partially protected by three injections of typhus tunica material in which there are moderate numbers of *Rickettsiae*, treated for from 24 to 48 hours with a 0.2 per cent formalin solution.

We believe that the immunization is due to the presence of the *Rickettsiae*, since in our preceding experiments we have satisfied ourselves that these organisms are the true etiological factors of the disease. For the reasons stated above, we believe that the formalinized vaccine does not contain living, but attenuated organisms, and that the immunizing effect is the result of treatment with formalin-killed *Rickettsiae*. This point, however, we admit, is not absolutely determined.

These experiments, together with the results obtained in the concentration of *Rickettsia* material by the diet method of reducing resistance as described in the paper which follows, furnish a hopeful method

We believe that the accumulated experience of the work of one of us with Batchelder, together with these two experiments, excludes the assumption that the complete immunity obtained in seven vaccinated animals in three separate, well-controlled experiments, and the comparative mildness of infection of three out of six vaccinated animals in another experiment were accidental, and we conclude that even with the relatively unconcentrated *Rickettsia* material used, it is

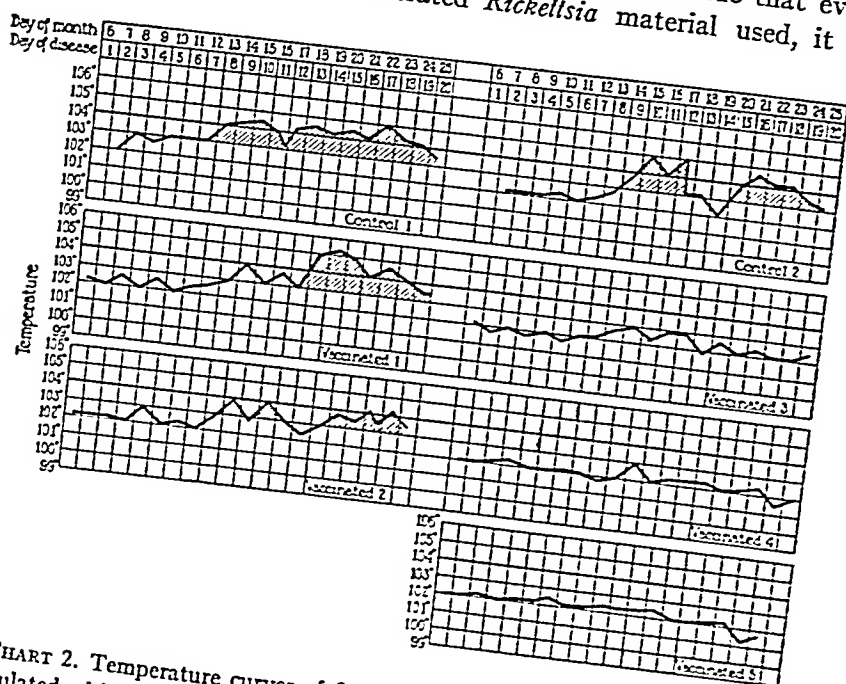


CHART 2. Temperature curves of five vaccinated and two control guinea pigs inoculated with 1 cc., respectively, of European typhus blood and brain.

possible to make a formalized vaccine which by itself will not produce typhus fever, but will confer either complete or partial protection. We are not yet sure what is the best interval for reinoculation after vaccination, nor do we know how long such artificially conferred immunity lasts. These important points will be the subjects of further reports.

A fundamental question is whether the vaccines which we had formalized and which we had kept only for from 24 to 72 hours in

and a reasonable theoretical basis for a procedure of active immunization against this disease in human beings.

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ported studies—in which we believe we proved the etiological significance of the *Rickettsiae*—our desire to obtain more regular results, particularly for vaccine production, led us to cast about for other methods of reducing resistance.

About 2 years ago, with Batchelder, one of the writers (2) endeavored to reduce the resistance of guinea pigs by feeding them on vitamin-free diets. The idea underlying this was the well-known fact that typhus epidemics have throughout history been most virulent and fatal in times of famine. While there are of course other factors besides nutritional deficiency which participate in rendering typhus particularly severe in times of hardship, war and crop failures, nevertheless, as analyzed by Hirsch (3) and subsequently by Kurschmann (4), the association with famine and consequent malnutrition appears to be one of the very important influences that contribute to the severity of epidemics. Moreover, there has been much experimental evidence in recent years to indicate that nutritional disturbances of various kinds exercise a beneficent influence upon susceptibility to various infections.

At the time when we first entertained the idea of applying this reasoning to typhus, we carried out a number of experiments which showed that animals fed on vitamin-deficient diets often responded to infection with severe and manifest illness, during which there was no rise of temperature, the temperature not infrequently dropping to subnormal within 2 or 3 days after inoculation, the animals sometimes dying on the 4th to the 8th day without having exhibited any of the characteristics of an experimental typhus. Owing to the fact that we were still using the Giemsa method of searching for *Rickettsiae*, we were not satisfied with the results of these experiments in regard to any evidence of increased development of *Rickettsiae*.

We resumed experiments of this nature in the course of the last year, making more careful *Rickettsia* studies by means of the formalin-buffer-methylene-blue-saffranin stain perfected by one of us and elsewhere published (5).

Experiments on Guinea Pigs

In accordance with the experience of Abels (6) and of several other investigators (7) in regard to the resistance-depressing effects of

STUDIES ON TYPHUS FEVER

VI. REDUCTION OF RESISTANCE BY DIET DEFICIENCY

By HANS ZINSSER, M.D., M. RUIZ CASTANEDA, M.D.,
AND C. V. SEASTONE, JR.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston)

(Received for publication, December 5, 1930)

The average guinea pig or rat possesses a resistance against typhus infection which limits the distribution of the virus and leads to almost invariable recovery of the animals. Many of our efforts during the last 2 years have focused upon the production of increased susceptibility, in order that we might convert the milder, experimental disease into a fatal one or at least obtain an increased yield of *Rickettsia* bodies for immunological studies.

As reported in a preceding paper (1), preparatory benzol injections into rats yielded us a sufficient number of successful experiments to furnish peritoneal exudates rich in *Rickettsiae* and suitable for the etiologial demonstration which we reported. Although we have at the same time studied cultural methods by the tissue-plasma technique and by the Tyrode-serum method of the Maitlands, and have obtained some multiplication, we continued to concentrate upon the susceptibility-enhancement experiments because—wherever successful—the peritoneal exudates of animals inoculated in the stages of depression yielded not only many intracellular, but plentiful extracellular *Rickettsiae* which were, therefore, easily freed of plasma and cells by simple washing in the centrifuge. The benzol method was successful in only about one out of three times, since individual differences between rats seemed to necessitate adjustment of time factors and the number and amounts of the benzol injections which could not be approximated without a very large series of experiments. Although we employed the benzol method, nevertheless, in the previously re-

peritoneal exudate was extraordinarily rich in *Rickettsiae*.* In all the other animals, without exception, *Rickettsiae* were found both in peri-

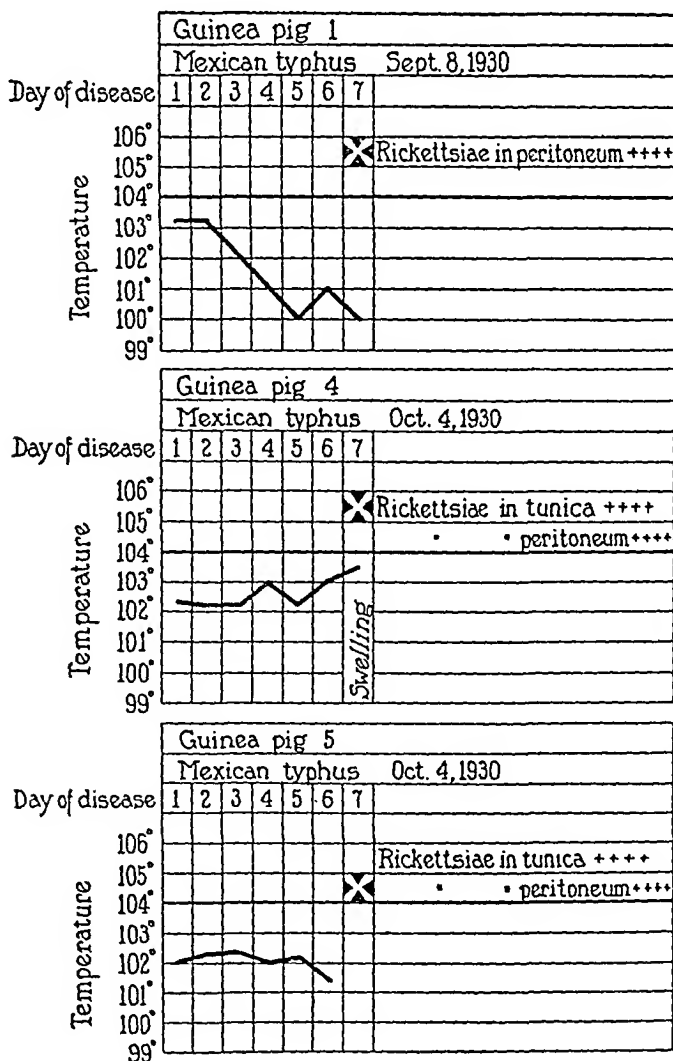


CHART 1. Temperature curves of three guinea pigs inoculated with Mexican virus from tunica material. In all of them, in spite of the absence of a characteristic typhus curve, there was serious illness and extraordinary numbers of *Rickettsiae* were found both in tunica and peritoneum.

* We do not include a photograph of the exudates containing the large numbers of *Rickettsiae* in these animals since they differed in no essential respects from similar exudates from the successful benzol animals, pictures of which were published in a preceding paper.

scurvy, we fed guinea pigs on a diet consisting of rolled oats, skimmed milk, autoclaved 1 hour at 15 pounds, and water. After anywhere from 14 to 20 days on such a diet, the guinea pigs showed signs of weakness, and sometimes joint swelling, and when one or another of the animals of each lot exhibited distinct symptoms of deficiency, we inoculated intraperitoneally with tunica material from Mexican typhus animals.

Altogether, we have in this way investigated thirteen guinea pigs, weighing about 250 gm. each.

Of this series, two animals were of little significance. They died without the development of temperature, both on the 6th day after inoculation, of secondary infection with bacteria. It is of some interest that in one of them on the 6th day, in addition to the bacteria there were numerous *Rickettsiae* in the peritoneal exudate.

Another animal of the series is of relatively little interest because it developed an irregular typhus fever, not in principle differing from the ordinary experimental disease. In this guinea pig the diet had obviously not been carried far enough.

All the rest of the guinea pigs, however, yielded results of the greatest interest to us in our efforts to produce vaccines for the practical application of the principles recorded in our Study V (8). In three, out of the ten remaining animals, the temperature curve showed a tendency to rise immediately, reaching between 104°F. and 105°F. on the day after inoculation, touching 105°F. in two of these on the 3rd day, and 106°F. in one of them on the 4th day. Examined on the 6th, 7th and 8th days, respectively, when the temperatures had returned to normal in all of them, there were plentiful *Rickettsiae* in the tunica. In the remaining seven guinea pigs, the temperature either did not rise at all, or went up very slightly on the 3rd or 4th day; in all but one of them it remained at or below 103°F.; in only one it rose to 104°F. for 1 day.

Two of these guinea pigs died, one on the day after inoculation, after a drop from a temperature of 102.4°F. to 101°F. Even in this animal a moderate number of *Rickettsiae* were found in the tunica. The other animal that died spontaneously on the 7th day showed a few small pneumonic lesions in the lungs, but the

SUMMARY OF DIET EXPERIMENTS

The above experiments demonstrate that guinea pigs and rats subjected to vitamin-deficient diets to a point at which deficiency symptoms appear, and then inoculated with typhus virus, exhibit clinical pictures which indicate a far more severe infection than that observed in normal animals after inoculation. There is also a wider distribution of *Rickettsiae* and a concentration of organisms which, in pleural and peritoneal exudates, amounts to almost cultural proportions.

Important from our point of view is the fact that these experiments furnished a step toward the accomplishment of our purpose, which was to obtain amounts and concentrations of *Rickettsiae* suitable for immunological studies until such a time when tissue culture may have developed to a practically useful stage.

The experiments are of immediate importance in that they furnish us a method for improving our technique of active immunization reported upon in the preceding paper, No. V (8).

From the epidemiological point of view these experiments at least suggest an explanation of one of the important factors which enter into the historical association of high typhus mortality with war and famine.

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toneum and in tunica in profuse, even cultural numbers, and in many of them—to our surprise—large numbers of the *Rickettsiae* were extracellular. Typical curves of such guinea pigs, in which large numbers of *Rickettsiae* were found, are shown in Chart 1.

Experiments on Rats

Rats were fed on slightly baked biscuits containing vitamin-free casein, vitamin-free lard and vitamin-free starch, salts and water, 5 to 7 gm. of this being fed daily. As a rule, the rats showed the effects of this diet within 14 days. At the time when the first effects of the diet appeared, the animals were intraperitoneally inoculated with tunica material from infected guinea pigs.

Of six animals so investigated, two were apparently too weak to stand the inoculation, and died without result.

The other four animals, however, showed a remarkable departure from the ordinary course of rat typhus. All of them became noticeably ill between the 4th and the 6th day after inoculation. Two were killed on the 5th and the 6th day after inoculation, when they were noticeably sick, both of them showing considerable numbers of *Rickettsiae* in the tunica and peritoneal exudate, and one of them having an abnormal amount of peritoneal and pleural exudate, in both of which enormous numbers of *Rickettsiae* were found. In this animal, also, scrapings made from the visceral pleura showed very large numbers of the organisms within the endothelial cells, and the *Rickettsiae* were also present in smears from the blood, the endocardium, the spleen, liver and pia mater.

A third rat very sick on the 4th day after inoculation, was killed and showed numerous intra- and extracellular organisms in the peritoneum, and large numbers were found in scrapings of pleural cells—indeed, more than were found in the peritoneum.

A fourth animal died spontaneously 6 days after inoculation. Again *Rickettsiae* were plentiful in peritoneal exudate and in the pleura. In all of these animals, with the exception of those especially mentioned, which were apparently too far gone with the diet before the inoculation was undertaken, infection was far more severe, and the yield of *Rickettsiae* extraordinarily increased over that usually observed after the inoculation of normal rats.

The present communication describes a test method for determining the presence or absence of a chill-producing factor in antipneumococcus serum (2), a study dealing with the effect of drugs on the reaction, and with the origin and some of the properties of the causative agent.

Test Method

In the first experiments, it was found that serum which had produced a typical reaction in a patient, produced a corresponding reaction when injected into the jugular vein of a dog. On the other hand, a serum which had failed to give a reaction in a patient, also failed to give one in a dog. Following these initial experiments, a considerable number of serums, which had been or subsequently were used in the pneumonia wards at Harlem Hospital, were tested. The experimental results were in striking agreement with the clinical ones.

The procedure in the animal test is as follows: The dogs selected are of the short-haired variety, of a weight between 5 and 10 kilos; the temperature is taken by rectum before and at 20 minute intervals after the serum injection, the thermometer being inserted the same distance for each reading; the serum is injected into the jugular vein, no local anesthesia or operative exposure being necessary.

The criterion of a positive reaction is a rise of temperature of 1.5°F. or more, occurring within 60 to 75 minutes, and maintained about an hour. A chill may or may not be present, apparently unrelated to the degree of temperature elevation. In a typical, strong reaction, there is first a definite chill, coming on within half an hour after the injection; the rise in temperature is observed about 10 minutes later, and reaches a maximum of 2°F. or more in about 15 minutes. The dose of the serum required to produce a positive reaction varies from 1 to 10 cc., practically the same as that required in patients. The occurrence and duration of a chill in the dog were found to be variable, so that the hyperpyrexia within 60 to 75 minutes was taken as the more reliable criterion. It was observed in many instances that whereas a small dose of serum would produce only a rise of temperature, a larger dose would result in both a chill and rise of temperature.

It should be pointed out that especial care must be taken to secure a proper test animal, since many dogs apparently suitable fail to give the expected response. Any dog that fails to respond to an established minimal effective dose of a known chill-producing serum should be discarded. Further if the dog reacts positively to a serum he is then given a serum known not to produce a chill in a patient; with no reaction from this he may be considered suitable as a test animal.

ON THE NATURE OF THE CHILL-PRODUCING PRINCIPLE IN ANTIPNEUMOCOCCUS SERUM

BY ALBERT B. SABIN AND GEORGE B. WALLACE, M.D.

*(From the Departments of Pharmacology and Bacteriology, University and Bellevue
Hospital Medical College, New York University, New York)*

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Following the intravenous injection of concentrated, antipneumococcus horse serum, there frequently occurs a systemic reaction of varying intensity and widespread effects. This comes on usually in from 30 to 60 minutes after the injection and begins almost always with a chill which is accompanied and followed by an elevation of temperature. The temperature rise covers a period of several hours. This reaction is quite distinct from an immediate anaphylactic effect and from delayed serum sickness. It is not peculiar to antipneumococcus serum since a similar reaction may occur from intravenous injection of whole blood, vaccines, various proteins and split protein products, nucleins, and such substances as salt, sugar, colloidal metals and even distilled water (1). The nature of the substance producing the reaction is not known, nor is the mechanism through which the effects are brought about, understood.

The favorable clinical results of non-specific protein therapy seem to be associated with the occurrence of the reaction, although it is impossible to state what particular changes induced are responsible for the beneficial effects. Whether in the use of a specific serum such as antipneumococcic serum, this common reaction is of additional value, is not so clear, since there is no satisfactory statistical or other evidence bearing on this point. Certainly in animals, the presence or absence of the chill-producing factor has no relationship to the protective power of the serum. It is considered by many to be a distinct disadvantage, on account of the additional strain an unusually high temperature, delirium and associated symptoms, may cause. This feeling is responsible for some limitation of a more general employment of the serum therapy.

CHILL-PRODUCING PRINCIPLE IN SERUM

TABLE I

Comparison of Animal Tests with Clinical Effects

Serum	Reaction in dogs		Reaction in patients	
	Dose	Effect	Dose	Effect
1	cc. 1		cc. 2	Definite chill
2	5	Severe chill; +2.2°F.	10	No chill
	5	None		
	10	"		
3	4	Marked chill; +3.3°F.	4	Marked chill
4	4	Chill; +1.5°F.	5	Chill
5	5	" +1.8°F.	5	"
6	4	" +2.0°F.	5	"
7	5	None	5	No chill
	10	"	10	" "
8	5	Chill; +1.6°F.	20	" "
9	5	None	5	Chill
	9	Chill; +1.8°F.	10	"
10	5	None		
	9	"	10	No chill
11	10			
12	10	+1.0°F.	10	Mild "
13	10	+2.8°F.	10	Chill
14	5	+2.7°F.	10	"
	10	+1.9°F.	5	"
15	10	Chill; +2.2°F.		
16	9	Slight chill; +2.1°F.	10	"
17	10	" " +2.1°F.	10	"
18	10	Chill; +2.0°F.	10	"
19	10	+2.0°F.	10	"
20	10	None	10	No chill
21	9	Slight chill; +1.7°F.	10	Chill
22	10	+1.0°F.	10	Mild chill
		None	10	" " in one; no chill in
23	3			another
	5	+1.2°F.	5	Marked chill
24	10	+2.0°F.		
25	5	None	10	No chill
	10	No change	10	Mild "
		+1.0°F.		

To determine whether the same test animal could be used repeatedly, the initial effective dose of a serum that was known to give chills in patients, was injected daily or every other day. One dog failed to respond with the sixth injection of a dose to which he previously responded with a chill and hyperpyrexia; when twice the dose was injected he again reacted. In another dog using a different serum, no tolerance was observed after ten injections. In the dogs that were used for routine testing, no appreciable tolerance was noticed after 5-7 injections; several dogs were used as many as 12-15 times. However when a dog has been used several times, it is advisable to control subsequent tests with a known serum. If the interval between injections was greater than 2 days, some dogs would develop immediate anaphylactic symptoms when they were subsequently used for testing; the shortest interval was 2 days. The anaphylactic symptoms consisted of collapse and marked gastrointestinal irritation, shown by retching, vomiting, and diarrhea. They came on immediately after injection and lasted from 2-20 minutes. If the serum used for the test contained the chill-producing factor, both the immediate anaphylactic and the delayed "chill" reactions could be observed; after the anaphylactic reaction, the chill-producing factor acted as it did originally. The two mechanisms are therefore quite distinct.

Table I gives a comparison of animal tests with clinical effects from twenty-five serums, some prepared by Dr. Banzhaf of the New York City Health Department and some by Dr. Felton.

The striking agreement seen in Table I indicates that the test shows not only the presence or absence of the chill-producing factor, but also that the result may be transferred directly to human beings. It may be observed, however, that patients are more sensitive than dogs. One serum producing no reaction in a dog, when tested on patients in the same dose produced a mild reaction in some and none in others; a similar result was obtained with a serum that gave a rise of only 1°F. in dogs. When a serum produces only a rise of temperature in a dog, the effect of the same dose in the patient is usually both chill and pyrexia. It was observed, however, that a serum which gave a properly controlled positive reaction in dogs, invariably produced chills in patients. In any case where a "borderline" result is obtained on dogs, the test should be repeated with a larger dose.

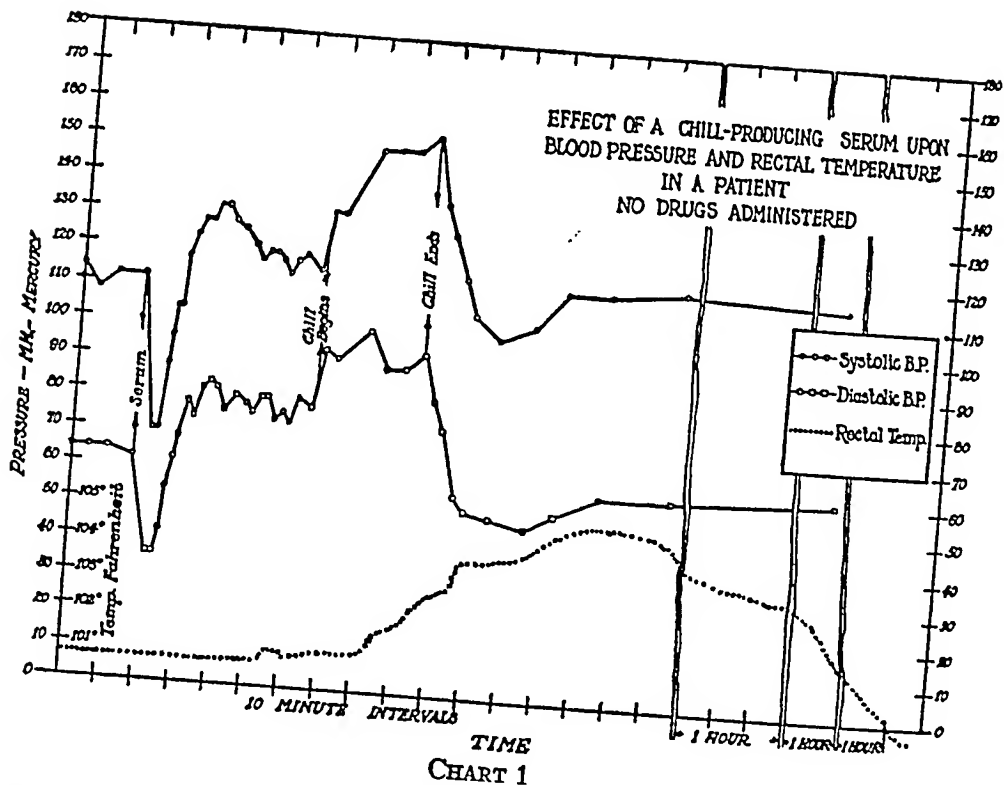


CHART 1

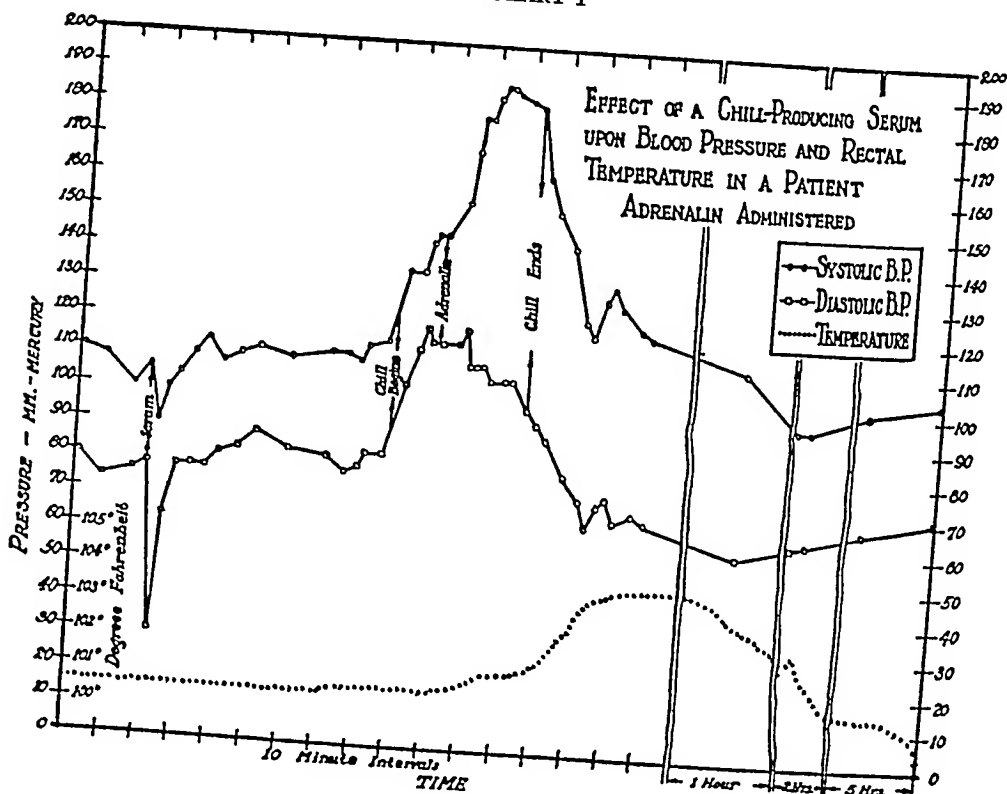


CHART 2

Effect of Drugs

The effect of drugs on this reaction was studied both in man* and dogs. The purpose of this study was first, to find a method of controlling the reaction and secondly, to obtain information on the mode of action of the chill factor from the known action of the drugs which modified it.

Adrenalin.—A routine treatment in certain hospitals has been the administration of 0.6 cc. of a 1:1000 solution of adrenalin subcutaneously. This practice was apparently based on the assumption that the serum reaction was anaphylactoid. The efficacy of adrenalin was never apparent, however, since the chill is self-limited regardless of treatment, and pyrexia always occurred. To determine whether or not adrenalin had any beneficial effect on the course of the chill or pyrexia, four patients were injected with the same dose of a chill-producing serum; two were untreated and two received adrenalin when the chill began. The rectal temperature was recorded every minute by an automatic electric recorder and the blood pressure was taken every 5 minutes. The data are presented in the accompanying charts (1 and 2).

While the injection of a non-chill-producing serum is not followed by any appreciable blood pressure or temperature changes, immediately following the chill-producing serum injection there is a fall in both the systolic and diastolic pressures. These return to normal within 2 minutes,† and the pressure then remains approximately level until the onset of the chill, when both the systolic and diastolic pressures rise to a maximum, maintained as long as the chill lasts. The abatement of the chill and the return of the pressure to normal occur practically simultaneously. The temperature begins to rise about 10 minutes after the onset of the chill and reaches a maximum about the time the chill stops. Fol-

* These and other clinical studies made by us were carried out in the Pneumonia Division at Harlem Hospital, through the courtesy of Dr. J. G. M. Bullock, Director. The observations recorded were on patients given the serum for its effects as a therapeutic agent.

† This initial fall in blood pressure apparently is not due to venepuncture since the pressure taken during venepuncture for withdrawal of blood showed no such fall. This initial fall occurred most frequently following the injection of chill-producing serums and only occasionally after the injection of a reaction-free serum.

in this manner four times, after which shivering stopped and the blood pressure gradually fell and remained below normal. (See Chart 3.) It was thus possible to break each beginning paroxysm by amyl nitrite, and to shorten the usual duration of the chill from 25 minutes in untreated patients and 30-35 minutes in adrenalin-treated patients to about 5 minutes. Amyl nitrite had no effect on the rise of temperature. These observations were repeated on other patients. Following this, amyl nitrite was administered as routine to all patients with a chill, and it was found to be of distinct benefit in mild and moderately severe chills, whereas markedly severe reactions were apparently not benefited, for even though the shivering would temporarily stop after amyl nitrite inhalation, it would immediately resume and continue, and the repeated inhalations would leave the patient with a severe headache. When no effect was evident after 5-10 inhalations at $\frac{1}{2}$ minute intervals, it was deemed advisable to discontinue further application.

When amyl nitrite was administered to a dog with a chill, it was usually possible to check it within 1-2 minutes; the rise of temperature was not prevented.

Antipyretics.—Acetphenetidin, 0.5 gm. given 45 minutes before the serum injections, and 0.15 gm. at half hour intervals afterward prevented the reaction in a dog. In a patient, however, 1 gm. in divided doses over a period of an hour and a half had no effect.

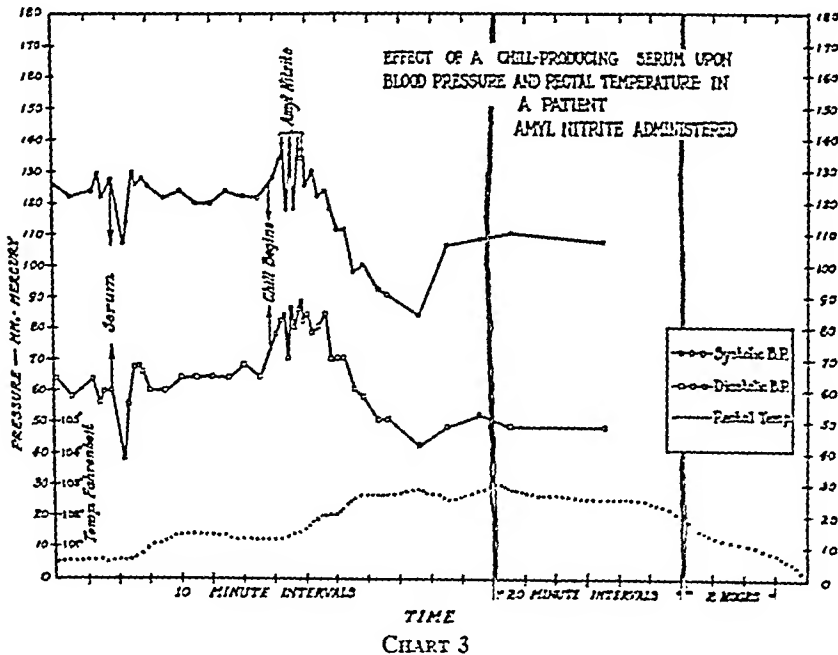
In two patients who received acetylsalicylic acid, 0.6 gm. in 1 ounce of whiskey, with external warmth to promote sweating, there was no chill and a markedly lessened temperature rise from a serum which gave typical reactions in untreated patients.

Opium and Morphine.—Patients given Dover's powder, 0.6 gm. 10 minutes before the serum injection had no chill and a negligible rise in temperature, whereas in untreated patients the same serum induced a typical reaction. In cases where a serum known to cause a very severe reaction was used, Dover's powder seemed to ameliorate but not prevent the symptoms.

In dog experiments morphine sulfate given subcutaneously about half an hour before the serum produced the following results: with a dose of 3 mg. per kilo, there was no chill and the temperature fell instead of rising; with 1 mg. per kilo there was no chill and the tem-

lowing the administration of adrenalin after the blood pressure has apparently reached its maximal level, a further rise is observed. The course was otherwise not perceptibly different in the patients who received adrenalin, except that the chill seemed to have lasted longer. Similar results obtained with other patients indicate that adrenalin is without any beneficial effect in the treatment of this reaction.

Amyl nitrite.—The coincidence of the rise and fall of blood pressure with the onset and abatement of the chill suggested the use of blood



pressure depressing drugs. Two patients were given an intravenous injection of a similar dose of the same serum that was used in the tests with adrenalin; the temperature and blood pressure were recorded as before. As soon as the patients began shivering and the blood pressure rose, amyl nitrite was administered by inhalation for 10-20 seconds. The shivering stopped and the blood pressure fell for about a minute and then returned to its previous level; amyl nitrite was then again administered with a similar effect; it was applied

that there is a disturbance of some coordinating central nervous mechanism. The fact that adrenalin may prolong the chill and amyl nitrite check it, whereas neither affects the temperature rise, and that a typical temperature rise can occur without a chill, makes it seem that the chill is a result of a vasomotor disturbance and is distinct from the temperature change. The temperature rise which is lessened by antipyretics and opium would appear to be due to a disturbance of the cerebral heat-regulative mechanism. It is impossible at present, however, to sharply define the areas involved.

Occurrence of the Chill-Producing Principle

Heterologous and Homologous Serums.—It may be stated at the outset that the chill-producing activity of horse serum, when injected into human beings or dogs, is not dependent on the fact that a foreign serum is employed. There are many recorded observations of chill reaction following the transfusion of homologous matched bloods, and also occurring in autoserotherapy and convalescent serum therapy. We have ourselves many experiments in which dog serum or defibrinated blood injected into dogs produced a typical reaction, in dosage corresponding to that of effective horse serum.

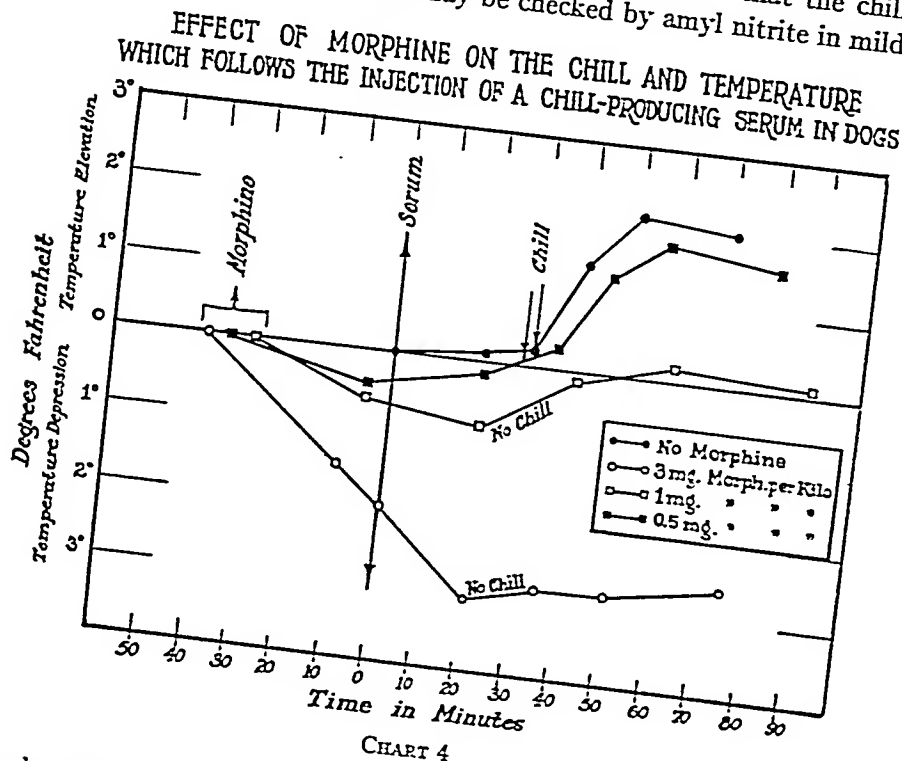
Inconstancy of Occurrence.—The occurrence of the chill factor in serum is not a constant phenomenon and different serums which do contain it vary markedly in their content. In the treatment of pneumonia with large doses of unrefined antipneumococcus serum, it was observed that the chill reactions did not occur with all serums (3). We have examined a number of antipneumococcus horse serums by means of the dog test and found that whereas 20 cc. of one serum was sufficient to produce a marked reaction, 60 cc. of another gave no reaction. Further we have found that normal horse serums behave similarly, some samples giving a reaction in 40 cc. dosage, and others no reaction in 65 cc. Finally in examining dog serums we have found the same variability, some giving a positive reaction with 40 cc., others giving no reaction with 100 cc.

Absence in Fresh Blood.—If blood from a dog whose serum is shown to give a positive reaction is injected immediately on drawing into a test dog, no reaction occurs. The following experiment is evidence for this: the bloods of the dogs selected showed no cross agglutination;

perature showed neither rise nor fall; with 0.5 mg. per kilo the serum caused both chill and a rise of temperature. These results, which were carefully controlled ones, are shown in Chart 4.

These morphine experiments have not been repeated on human beings and consequently the effective dose here is unknown.

The practical conclusion from these experiments is that the chill but not the temperature rise may be checked by amyl nitrite in mild



and moderately severe, but not in the more severe cases; the chill may be prevented and the temperature rise made negligible by acetylsalicylic acid and whiskey, and by opium in all except the most severe cases.

When one considers the multiplicity of widespread effects that accompany the reaction,—the chill, pyrexia, blood pressure changes, leucocytosis, metabolic changes, etc.—it is hard to escape the belief

TABLE II

Effect of Dog's Serum, Defibrinated Blood, Citrated Plasma and Fresh Whole Blood

Dec. 26, 1929			Jan. 1, 1930		
Time	Temp.		Time	Temp.	
	°F.			°F.	
10:44 a.m.	102.5	50 cc. serum from blood drawn on Dec. 24 injected intravenously	1:00 p.m.	102.7	45 cc. serum from blood drawn on Dec. 31 injected intravenously
10:49-53			1:05-08		
11:02	102.5	Shivering begins " continues " stops	1:25	102.6	Shivering begins " continues " stops
11:18			1:30		
11:20	102.9		1:38	103.4	
11:35			1:50		
11:41	105.0		2:05	105.3	
Jan. 3, 1930			Jan. 4, 1930		
11:50 a.m.	103.1	80 cc. whole blood injected intravenously immediately on withdrawal	10:30 a.m.	102.9	80 cc. blood defibrinated with glass beads filtered injected intravenously within 15 min. of withdrawal
11:55-58			10:34-37		
12:15 p.m.	103.3	No shivering	10:53	102.7	Shivering begins " stops
12:28	103.5		11:00		
12:38	103.3		11:15	104.1	
12:52	103.5		11:35	105.5	
1:11	103.2				
3:45	102.8				
Jan. 6, 1930			Jan. 7, 1930		
2:30 p.m.	103.2	50 cc. citrated plasma injected intravenously. Immediate rigor, lasting 2 minutes	12:30 p.m.	102.5	50 cc. 3 per cent sodium citrate injected intravenously. Immediate rigor, lasting 2 minutes
2:32-36			12:40-44		
2:50	103.1	Shivering begins " stops	1:01	101.6	No shivering
2:55			1:18	102.4	
3:12	105.2		1:33	102.4	
3:38	106.0		1:57	102.4	

The same donor and recipient dogs were used in this series of experiments. In the experiment of Jan. 3, the 80 cc. whole blood was drawn from the donor and injected into the recipient in 20 cc. lots. In the experiment of Jan. 6, the blood from the donor was received in four centrifuged tubes, coated with paraffin, each tube containing 2 cc. of 30 per cent sodium citrate. The tubes were then centrifuged and 50 cc. of plasma obtained for injection into the recipient.

the donor weighed 20 kilos, the recipient was a small fox terrier, weighing 7 kilos, and selected according to criteria previously described. 45 cc. of the donor's serum gave a positive reaction on three occasions before this experiment. The dogs were placed next each other and blood taken from the donor in 20 cc. amounts was immediately injected into a vein of the recipient, less than a minute being required for each injection. A total of 80 cc. was injected in this manner. The recipient dog had no reaction from this injection. Subsequent injections of defibrinated whole blood and of citrated plasma from the same donor into the same recipient gave definitely positive reactions (Table II).

Effect of Refinement.—As has been pointed out, large amounts of unrefined pneumococcus horse serum do not always produce a chill reaction. When different lots of antipneumococcus serum or plasma are refined and concentrated by a uniform procedure, the resulting preparations, containing the total water-insoluble globulin, also vary in regard to their chill-producing properties, some giving reactions in doses of 1-10 cc., others being reaction-free in from 20-30 cc. amounts. To determine what part the procedure of refinement played in this variability, comparisons were made of serums before and after refinement. Eight different lots of antipneumococcus serum and plasma (each lot containing more than one bleeding from the same horse or the bleedings from more than one horse) and the refined and concentrated globulin solutions prepared from them* were tested for the content of chill-producing principle. The results are given in Table III.

It may be seen from the table that the chill-producing activity of the different lots of original serum varied considerably; whereas 20 cc. of one serum was sufficient to produce a marked reaction, 60 cc. of another serum gave no reaction. It may also be observed that the dose of concentrated solution required to give a positive reaction is roughly proportional to the chill-producing activity of the serum from which it was prepared. From these results it may be concluded (1) that the chill-producing principle was present in the original serum and not developed during the process of preparation, (2) that it was

* These were prepared by Dr. Banzhaf, using the ammonium or sodium sulfate method of separation. *Proc. Soc. Exp. Biol. and Med.*, 1925, 22, 329.

concentrated with the water-insoluble globulin (soluble at 30 per cent saturation of $(\text{NH}_4)_2\text{SO}_4$ and 12 per cent Na_2SO_4 ; insoluble at 50 per cent saturation $(\text{NH}_4)_2\text{SO}_4$ and 20 per cent Na_2SO_4), and (3) that it may be possible to predict the chill-producing activity of the total water-insoluble globulin fraction from the original serum.

Effect of Immunization.—During the process of immunization large amounts of dead bacteria are injected into the horses and products of these might be considered as a possible source of chill-producing activity. The fact that the horses are not bled till at least a week after the last immunization treatment, and that the horses being immune, the bacterial products are more rapidly destroyed, renders this possibility improbable. However, to determine this point positively, it would be necessary to test the serum of the same horse before and after the injection of bacteria. Since facilities for carrying out such a test were not available, the serums (without preservative) of six normal horses were studied instead. It was found that normal horse serum may give a (positive) chill reaction indistinguishable from that produced by antipneumococcus serum, the minimal effective dose varying from 40 cc.—90 cc. From these data it may be concluded that the immunization treatment is not necessary to render a serum chill-producing. It is impossible to state whether or not it affects the minimal effective dose for a chill-reaction, but since the variation in normal serums is considerable, it would seem that other factors, at present unknown, influence the chill-producing activity of a serum to a greater degree.

Nature of Chill-Producing Principle

Toxic Substances in Blood.—When fresh, defibrinated, homologous blood is injected into rabbits or cats, severe poisoning ensues. The mechanism of this has been studied by Freund (4). If the blood is injected within a few minutes after drawing, death results, in rabbits through cardiac paralysis, in cats through respiratory stoppage. If defibrinated rabbits' blood is allowed to stand for 15 minutes before injecting, it is much less toxic, and after 24 hours, it has no appreciable action. Freund believes that these different effects are due to two sets of substances, "early" poisons which lose their activity in a short time, and are replaced by "late" poisons, which are much less intense

TABLE III

Comparison of Original with Refined Serum

Preparation No.	Dose of original serum or plasma injected	Reaction	Salt used for fractionation	Concentration by volume, i.e., vol. of origin serum vol. of antibody glob.	Dose of concentrated preparation injected	Reaction
1	30 cc. of serum and plasma mixed	Chill; +2.6°F.	(NH ₄) ₂ SO ₄	8	cc. 10	Marked chill; +2.2°F. +1.8°F.
2	20 cc. of plasma (40 cc. " "	No reaction " "	(NH ₄) ₂ SO ₄	9	5	No reaction
3	40 cc. Horse 1, 3/13/29* 40 cc. " 1, 12/1/28	+1.3°F. +1.0°F.	NH ₄ SO ₄	5	10 10 (another dog) 5	Mild chill; +1.5°F. No " +1.5°F. No reaction
4	40 cc. plasma Horse 2 40 cc. serum " 3 Serum Horse 4, old, decomposing	No reaction " " Not tested	(NH ₄) ₂ SO ₄	10	10 9 5	+2.1°F. Chill; +1.5°F. No reaction
5	65 cc. Horse 1, 5/6/29 50 cc. " 5, 7/11/28	+2.2°F. +2.2°F.	NH ₄ SO ₄	10	6	+2.4°F.
6	60 cc. " 5, 8/8/29	+2.6°F.	NH ₄ SO ₄	10	7	Slight chill; +2.8°F.
7	20 cc. " 5, 6/13/29 7/25/29	Slight chill; +3.0°F.	NH ₄ SO ₄	10	5	+3.1°F.
					1	No reaction

*These experiments were carried out during October and November 1929. The dates in this column are those on which the horses were bled.

was drawn into paraffined centrifuge tubes containing sufficient 30 per cent sodium citrate to make the final concentration 3 per cent. The blood cells were centrifuged and the clear plasma was injected. Following the injection an immediate rigor occurred, rendering the dog entirely stiff for 2 minutes; the typical chill and temperature reaction was not prevented. To show what part the citrate *per se* played in the entire reaction, 50 cc. of 3 per cent sodium citrate in physiological saline was injected into the same dog the following day. The rigor again occurred immediately after injection, but there was no chill or rise of temperature. (See Table II.)

Similarly, the effect of sodium fluoride was studied on account of its property of preventing certain enzymatic changes in drawn blood. Blood was mixed with sodium fluoride (300 mg./100 cc.), the cells were centrifuged, and then sufficient calcium chloride added to precipitate the excess of fluoride. The plasma either clotted, or a fibrin-like precipitate formed. The serum which separated was used for testing; no appreciable difference was observed between the reaction produced by this serum and that obtained by defibrination with glass beads.

It is thus evident, that whatever the nature of the chill-producing principle in dog blood, it is formed in the blood regardless of whether or not coagulation takes place and is not affected by sodium citrate or sodium fluoride in the quantities used.

Effect of Filtration.—Drinker and Brittingham (8) investigated the reactions which followed the transfusion of human citrated blood. Their criterion of a positive reaction was a rise of temperature of 2.5°F. in the patient. They concluded that although serum was toxic, citrated (1-2 per cent) plasma which was thoroughly freed from cellular constituents, either by rapid centrifugation or Berkefeld filtration was reaction-free, and that the reactions following the transfusion of human citrated blood were related to the changes which the sodium citrate produced in the red blood cells, promoting hemolysis, and to changes in the plates, part of the process of coagulation. In our own experiments the injection of centrifuged or Berkefeld filtered citrated plasma still gave positive reactions. These results taken together with the known preserving property of sodium citrate seem to indicate that the principle we are dealing with is not of cellular origin.

in their action, and lose their effectiveness in 24 hours. These poisons according to Freund arise from the destruction of blood cells and especially of blood platelets.

The chill principle appears to be different from the poisons described by Freund. In our experiments described above, dogs' blood injected immediately after withdrawal failed to produce the chill reaction. On the other hand dog serum injected at intervals from 15 minutes to 7 days after drawing, gave the reaction. In those cases where defibrinated dog blood failed to give the reaction, the serum, citrated plasma, and physiological salt solution extracts of the clot, ground up with sand also were negative. That the negative serum contained no antagonistic substance is shown by the fact that it failed to inhibit a positive reaction when mixed with a chill-producing serum before injection. It would appear from these experiments that the chill-producing principle is not always present in blood but may be formed when the drawn blood is allowed to stand.

Starling (5) and others have shown that freshly defibrinated blood exerts a strong vasoconstricting action on the kidney and liver vessels. Bodo and Marks (6) have shown that this action can be removed by perfusing the defibrinated blood through the lung for 20-30 minutes. Through the kindness of Dr. Bodo, dog's blood known to give a positive reaction, was perfused through the dog's own lungs for a period of more than 45 minutes and subsequently tested. The chill reaction following the injection of the perfused blood did not differ from that produced by the original defibrinated blood. It may be concluded therefore that the vasoconstrictor substance mentioned plays no essential part in the chill reaction.

Effect of Anticoagulants.—Studies by Loucks and Scott (7) on the effect of different anticoagulants showed that citrate not only prevented the fall in surface tension which is usually observed in shed blood but actually raised it. Strong solutions of sodium citrate (3 per cent) are known to prevent to a great extent the disruption of white cells and platelets which occurs in drawn blood. This suggests that citrate prevents at least some of the changes which occur in blood after shedding. The following experiment indicates, however, that sodium citrate is without effect in inhibiting the changes which are responsible for the formation of the chill factor in certain bloods. Blood

are shown in Table IV. That a simple procedure like desiccation *in vacuo* is capable of endowing certain protein solutions with the property of producing chills and pyrexia, and probably the other associated systemic changes as well, is of great physiologic interest.

Relation to Fibrinogen.—Kyes (13) believes that the chill-producing activity of fowl serum is due to residual fibrinogen. The observation that plasma is no more active than serum, that certain plasmas and serums may be injected without any reaction, casts some doubt on this assumption. In the refinement of antipneumococcus serum or

TABLE IV

Reactions of Inactive Globulin Antibody Solutions after Desiccation in Vacuo

Dog	Preparation	Treatment	Date of injection	Dose	Reaction
1	52	Original	6/14/30	cc.	
		Desiccated April '30	6/16/30	10	None
		" 6/17/30	6/18/30	10	Positive (chill; +2.6°F.)
		Original 6/20/30	6/20/30	10	" (" +2.4°F.)
2	34	"	6/21/30	10	None
		Desiccated	6/27/30	10	Positive (+2.5°F.)
3	35	Original	6/23/30	10	None
		Desiccated	6/27/30	10	Positive (+2.0°F.)
		Original	6/28/30	10	None
		Acid-globulin fraction of desiccated	7/ 2/30	12	Positive (+1.9°F.)
		Alkaline globulin fraction of desiccated	7/ 3/30	18	None

plasma by $(\text{NH}_4)_2\text{SO}_4$ the precipitate obtained with 30 per cent saturation, containing fibrinogen along with so-called euglobulin, is discarded; still the globulin obtained by further saturating the supernatant fluid with $(\text{NH}_4)_2\text{SO}_4$ (up to 44 per cent or 50 per cent saturation) is known to be chill-producing. We have carried out the following experiment: fibrinogen was precipitated from antipneumococcus plasma by half saturation with NaCl; the precipitate was taken up with 1 per cent NaCl in one-sixth the original volume. 10 cc. of this fibrinogen

Effect of Heat.—Zinsser (9) showed that the principle in goat serum which is toxic for rabbits is destroyed by heating at 56°C. for 30 minutes. The toxicity of the globulin fraction of guinea pig serum for guinea pigs (Bordet (10)) is unimpaired when heated to 60°C., but is destroyed by heating at 70°C. for 30 minutes (11). That the chill factor in antipneumococcic serum is not destroyed by heating at 56°C. for at least an hour is evident from the fact that this procedure, commonly used before the refinement and concentration of the serum, is without effect. Temperatures higher than 60°C. not only destroy the antibody, but also cause coagulation of the proteins (the concentrated globulin preparations coagulate more readily than the original serum). Dr. Falk (12) has evaporated antibody solutions to dryness *in vacuo* at a temperature under 40°C. with a subsequent heating to 80°C. for 1 hour and 90°C. for 30 minutes without any appreciable effect on the antibody content; our tests on Dr. Falk's preparations showed that the chill-producing activity was not destroyed. It appears then that heating serum at 56°C. for 1 hour, or heating the evaporated serum at 80°C. for 1 hour does not destroy the chill principle.

Effect of Dialysis.—In the preparation of refined pneumococcus antibody, the total water-insoluble fraction is sometimes obtained by dialyzing the serum against running tap water for several days; this does not appear to affect the chill-producing properties of the preparation. Our tests on globulin solution, which Dr. Banzhaf subjected to pressure dialysis against running tap water or distilled water for many days, showed no appreciable influence of this procedure on the chill-producing principle.

Effect of Desiccation in Vacuo.—Dr. Falk has developed a technique for desiccating pneumococcus antibody solutions *in vacuo* at a temperature below 40°C. In the course of tests for chill-producing properties of solutions prepared for us by Dr. Falk, the interesting observation was made that an antibody solution which originally gave neither chill nor temperature rise in a certain dose, produced a positive reaction in the same dose after desiccation. Further, it was noted that this apparently newly acquired property is associated to a greater extent with the acid than with the alkaline globulin fraction.* The data

* The nature of the fractions is described later on in this paper.

FRACTIONATION EXPERIMENTS

We have attempted to separate the chill principle from the antibody substances by changing the neutral salt and hydrogen ion content in "antibody solutions" prepared from antipneumococcus serum by either the sodium sulfate or the ammonium sulfate method. In the former the fraction insoluble in 12.5 per cent Na_2SO_4 was discarded, that precipitated at 20 per cent being retained; in the latter the fraction insoluble in 30 per cent $(\text{NH}_4)_2\text{SO}_4$ was discarded, that precipitated at 30-50 per cent retained. The resulting "antibody solutions" contained 1 per cent sodium chloride and their pH was about 6.8.

A uniform procedure for fractionation was used throughout.

To one volume of antibody solution, 2.3 volumes of distilled water were added, this reducing the sodium chloride to approximately $N/20$. A voluminous precipitate invariably resulted, precipitate A. Sufficient acetic acid was now added to bring the suspension to pH 4.8-5, leaving precipitate B. After standing for from 2-4 hours the suspension was centrifuged. The material thrown down was neutralized by $N/10$ NaOH and dissolved in 1 per cent NaCl to the original volume of the antibody solution, which it then resembled in physical appearance. This solution was designated "acid-globulin fraction." The supernatant fluid after centrifugation of precipitate B was further diluted with water to bring the NaCl concentration to approximately $N/80$ and then adjusted to pH 6.8. This mixture was left in the ice box overnight. The precipitate C was collected by centrifugation and was dissolved in 1 per cent NaCl solution also to the original volume of antibody solution. The solution was water-clear and was designated "alkaline globulin fraction."

Precipitate A mentioned above as produced when the NaCl concentration of the original antibody solution is reduced from 1 per cent to $N/20$ is not identical with precipitate B which is obtained when the further step of changing the pH from 6.8-5.0 is carried out. Thus when precipitate A is removed by centrifugation, and the supernatant fluid acidified to pH 5 a second precipitate, fraction γ , usually forms. Precipitate A suspended in $N/20$ NaCl, partially dissolves at pH 5.0. Precipitate A therefore contains two fractions; fraction α insoluble in $N/20$ NaCl at pH 6.8 but soluble at pH 5, and fraction β insoluble in $N/20$ NaCl at pH 6.8 and also at pH 5. Precipitate B suspended in $N/20$ NaCl dissolves almost entirely at pH 6.8. It contains fraction β of precipitate A, and also fraction γ which is soluble in $N/20$ NaCl at pH 6.8, but insoluble at pH 5. The acid globulin fraction therefore consists of fractions β and γ , and each of these has chill-producing properties. Fraction α on the other hand reacts like the alkaline globulin fraction.

solution (equivalent to the amount of fibrinogen in 60 cc. of plasma) was injected into dogs without any reaction. It seems to us, therefore, that fibrinogen is not the causative agent; nevertheless, Kyes states positively that treating fowl serum with a certain cephalin- CaCl_2 mixture to remove all fibrinogen renders it reaction-free. It appeared possible, however, that although the fibrinogen was not the causative agent, the treatment of the serum might be effective. Dr. E. Banzhaf (14) added the cephalin- CaCl_2 mixture to concentrated antibody solution (prepared from horse serum) without any apparent effect on its chill-producing properties on patients. We have repeated this experiment, using a dog as test animal with a similar result. On the other hand the serum of blood drawn from three dogs into this cephalin- CaCl_2 mixture failed to give any reaction, whereas ordinary serum from the same dogs gave a thermal reaction. The effect of cephalin and CaCl_2 should therefore be investigated further.

Relation to Lipoids.—Felton (15) comparing a number of pneumococcus antibody solutions observed that the chill-producing solutions were associated with a high phosphorus content, whereas non-chill-producing solutions had little or no appreciable amount of phosphorus. After removing from the total water-insoluble globulin a protein fraction which is insoluble at a salt concentration of $\text{N}/20 \text{ NaNO}_3$ and pH 4.6-4.8 and which contains most of the lipins, as well as phosphorus, the resulting solutions contained little or no chill-producing activity when used in patients. Felton concludes that there is an indication, therefore, "and an indication only, that this untoward reaction is associated with a phosphorus-containing substance."

Greenwald and Levy (16) have developed a method for preparing lipoid-free serums on a large scale; by this method the lipoid-phosphorus is reduced from 5.0 mg. per 100 cc. of original to 0.5 mg. or less in the extracted serum. Two serums from which the lipoids were thus extracted, were subsequently concentrated by the $(\text{NH}_4)_2\text{SO}_4$ method; we found that the resulting antibody solutions were chill-producing (in patient and dogs) to the same extent as the solutions prepared from the untreated serum. It appears therefore, that the chill reaction is not dependent on the amount of phospholipins present in the serum.

Felton has shown that removal of the so-called acid globulin results in solutions which are comparatively free from chill-producing activity. He is in doubt, however, as to whether this result is due to the acid treatment or whether the acid globulin itself is the chill producing substance. Our experiments confirm his and throw further light on the matter. They show, for example, that while removal of the acid globulin fraction is responsible for the decreased chill-producing activity of the original solution this is because the acid globulin precipitate carries with it in active form the chill-producing principle. They show also that the chill-producing activity of the acid globulin is not associated with its phospholipin content. Further the acid globulin

TABLE VI
Relation of Globulin Fractions to Protective Action

Preparation	Fraction	Mg. N/cc.	Protective units/cc.	Protective units per mg. N
38	Original	5.1	500	100—
	Acid-fraction	1.2	50	40+
	Antibody-globulin	1.8	200-500	110-275
37	Original	8.16	800-1000	100±
	Acid-fraction	1.74	10	5+
	Fraction soluble at pH 5.0, insoluble at N/80 NaCl at pH 6.8 and at pH 5.8	3.54	800-1000	225±
	Final supernatant	2.20	5	2+

produces a reaction only when it is derived from a solution itself chill-producing. It would seem probable then that the acid globulin is not itself the chill-principle but is associated with it as a carrier or adsorptive agent. An incomplete adsorption may explain the fact that the removal of the acid globulin does not remove all of the chill-producing activity.

Antibody Content of Fractions.—Table VI shows the data of mouse protection tests and nitrogen determinations on antibody solutions prepared by the ammonium sulfate or sodium sulfate methods and on the two globulin fractions isolated as previously described. These determinations were made for us in the Harriman Research Laboratory

Chill-Producing Properties of Fractions.—The acid and alkaline globulin fractions were compared by animal tests with the original antibody solutions in regard to chill-producing properties. The results are given in Table V. This table shows that the acid fraction

TABLE V
Relation of Globulin Fractions to Chill Production

Preparation	Method used in obtaining original antibody solution	Fraction*	Dose	Chill or thermal reaction
48	Na ₂ SO ₄	Original antibody	5	Positive
		Acid-fraction	7	"
		Antibody-globulin	10	Negative
G	Lipoids extracted (NH ₄) ₂ SO ₄	Original antibody	6	Positive
		Acid-fraction	6	"
		Antibody-globulin	12	Negative
		"	18	Positive
38	Na ₂ SO ₄	Original antibody	10	"
		Acid-fraction	10	"
		Antibody-globulin	20	Negative
37	(NH ₄) ₂ SO ₄	Original antibody	10	Positive
		Acid-fraction	15	"
		Antibody-globulin	10	Negative
		"	20	Positive
34	Na ₂ SO ₄	Original antibody	10	Negative
		Acid-fraction	15	"
		Antibody-globulin	15	"

* Acid-fraction refers to the fraction insoluble at N/20 NaCl concentration and pH 5.0.

Antibody-globulin refers to the fraction insoluble at N/80 NaCl concentration and pH 6.8 after removal of the acid fraction.

The volume of the various fractions is equivalent to that of the original antibody solution.

from a chill-producing solution gives a positive reaction, while that from a non-chill-producing serum is negative in this respect. Further the globulin solutions after removal of the acid fraction in each instance were reaction-free in the same or twice the original acting dose.

7. By changes in the sodium chloride and hydrogen ion concentration in antibody solutions an acid globulin and an alkaline globulin fraction may be obtained. The acid globulin fraction, whether or not phospholipin is present, contains the greater part of the chill-principle and a small part of the antibody substance; the alkaline globulin fraction contains the greater part of the antibody substance and a smaller part of the chill-principle. The acid globulin fraction is not itself the chill-principle but serves as a carrier of this, probably through an adsorptive process.

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through the kindness of Dr. K. G. Falk. Upon examination of the nitrogen figures, particularly those of Preparation 38, one notices that about 40 per cent of the nitrogen remains in the supernatant fluid to be discarded after the final precipitation at $N/80$ NaCl and pH 6.8. Further dilution of this supernatant fluid did not produce any appreciable precipitation; when it was acidified to pH 5.8-6.0, however, a precipitate appeared, which redissolved almost entirely at pH 5.0. This additional precipitate was included with the "antibody globulin" in Preparation 37. This protein, insoluble at $N/80$ NaCl and pH 5.8-6.0, was isolated from another chill-producing preparation and did not show any appreciable chill-producing activity.

Our results agree with Felton's observation that the acid-globulin fraction contains comparatively little antibody. It is thus apparent that whereas the chill-producing substance is associated to a greater extent with the acid-globulin fraction, the "protective antibody" is largely in the more alkaline globulin fraction.

SUMMARY

1. By means of a test method, which is described, the presence or absence of the chill-producing principle in antipneumococcus serum may be determined.

2. This principle may be present in normal blood or serum, homologous as well as heterologous, and the measures employed to obtain an antibody solution are not essential factors in its formation.

3. The chill reaction may be mitigated by the administration of nitrites and by opium and antipyretics. It is doubtful whether this fact offers any practical therapeutic application.

4. The reaction has no relationship to anaphylaxis or to certain toxic effects of drawn blood which have been studied by Freund, Starling and others.

5. The chill-principle appears to be formed only in blood which has been allowed to stand. Our experiments do not show that the reaction is dependent on formed elements, fibrinogen, or lipoids.

6. Anticoagulants, filtration, dialysis and moderate heating are without effect in removing the principle from the solution containing it.

The amount of bacterial suspension used in each case is noted in the tables. The method of obtaining the suspension has been explained previously.¹ 0.015 cc. of bacteria in 3 cc. of saline was used unless otherwise noted. From time to time, as the stock organism lost its toxicity, a change in the dosage injected became necessary. Whenever long intervals elapsed between experiments, the organism was always found to be less potent and frequently it became necessary to resort to animal passage to enhance its virulence.

The methods of obtaining filtrates, the average weights of the pigs, the type of broth used and its formula were all those described in our previous communication.¹

Protected pigs were animals which had had broth compresses applied to their abdomens for 48 hours previous to any injection. When mustard plaster was used for protection, strips of prepared commercial mustard plaster were cut, soaked in water and applied to the shaved abdominal wall for 5 minute intervals four times a day for 2 days (8 a.m., 12 p.m., 4 p.m. and 10 p.m.). When animals were treated with saline compresses, the gauze applied was soaked in saline and kept moist for 2 days. Compressing with meat broth extracts, peptone 1 per cent or 10 per cent (respectively) consisted in the local application for 48 hours of gauze soaked with these substances.

Specific filtrate was obtained in the manner described in our previous experiments. In some experiments, bacteria were added to the specific filtrate instead of to saline and the resultant suspension injected subcutaneously into non-protected pigs. Comparative experiments were carried on at the same time with pigs which had been previously protected by broth compresses.

The Lesion

Originally,¹ we described the lesions in terms of one, two and three pluses, representing the end results of the experimentation. In reviewing and again studying the data of these experiments, it became clear that we might adopt a different and more obvious standard of reaction, devoid of any element of the personal equation. Most guinea pig deaths occurred during the first 24 or 48 hours after the bacterial injection and practically all deaths occurred amongst those animals which showed a diffuse swelling of the entire abdominal wall on the 1st day. It was rare that pigs which had a localized inflammatory reaction died even though an abscess might form which later would slough, leaving what we originally described as a three plus end result. Sloughing did not necessarily have any effect on the life of the pig, but inasmuch as it occurred in practically all pigs with a diffuse swelling, the majority of which had a wide-spread ulceration or died, we had previously used its occurrence as a method of meas-

FURTHER EXPERIENCES WITH NON-SPECIFIC LOCAL CUTANEOUS IMMUNITY TO STAPHYLOCOCCUS AUREUS

LOCAL NON-SPECIFIC PROTECTION

By JOHN A. TOOMEY, M.D., AND S. O. FREEDLANDER, M.D.

(From the Divisions of Contagious Diseases and Surgery, Cleveland City Hospital,
and the Departments of Pediatrics and Surgery, Western
Reserve University, Cleveland)

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We have shown previously¹ that plain broth, when used as a skin compress, protected guinea pigs against the effects of subcutaneous injections of lethal doses of *Staphylococcus aureus*. These unspecific compresses protected as efficiently as those made with specific broth filtrates. Whatever production was accomplished either by plain or specific broth filtrates, was localized to the area compressed and lasted at least 24 hours after the removal of the compress. A definite histological difference was shown between the skin of the broth compressed and that of the non-broth compressed animal.

The following experiments were performed (1) to ascertain if the application of other types of local non-specific dressings brought about the same or a modified protection similar to that resulting from broth compresses and (2) to ascertain, if protection did occur, whether the histological picture was comparable to that found in our previous studies with broth-protected animals.

Methods and Materials. Definitions

The animals used were guinea pigs. All were shaved before compresses were applied except where otherwise noted. Because of the possible reaction to the simple act of shaving and irritation, the effects of such preparatory actions were likewise investigated. (Scraping the skin with a scalpel after shaving was considered "irritation.")

¹ Freedlander, S. O., and Toomey, J. A., *J. Exp. Med.*, 1928, 47, 633.

7. When injected subcutaneously, plain broth and specific broth were absorbed without a trace in less than 24 hours. When unprotected animals were injected with combinations of specific broth filtrate and bacteria, the animal was not protected by specific filtrate, but showed the same reaction as the unprotected pig injected with ordinary solutions of bacteria and saline (Table VII).

These last experiments are in contrast with the constant protection obtained in the controls by the application of broth for 48 hours previous to injection.

8. Protection from broth compresses lasted at least 120 hours (Table VIII).

9. When animals were injected with saline suspensions of bacteria and an effort was made to protect them with local broth applications after this injection, the mortality rate was extremely high as compared with that in the control pigs (Table IX).

10. Pigs which recovered from injections of staphylococcus suspensions were again injected with the same organisms within 30 days after the clearing of the previous lesion. Such reinjected pigs seemed still to have some protection (Table X).

II. Microscopic Results

Microscopically, the slides from the control animal treated with broth showed the same picture described in our previous article. When any agency protected the animal as well as broth did, the local reaction was the same as that in broth-protected animals. It could be said in general that any protective dressing used, produced a histological picture somewhat roughly proportionate to its ability to stimulate the tissue. Where complete protection occurred as with horse serum, plain broth and Liebig's extract, the same histological picture was produced as with specific broth or non-specific broth.

In the main, simple bandaging, plain water and saline compresses gave rise at best to but a slight reaction, a moderate increase in the clasmatoocytes and a barely noticeable thickening of the epidermal coats. Where protection was definite with these mild stimulants, the pathological picture was the same as though broth had been used. Examination of slides taken at various intervals after protection showed

urement. This measurement of reaction ignored other available differences.

After injections into the abdomen, there were obvious early and very striking local differences between the protected and non-protected pigs. This local difference in early reaction was most important as far as the pig's life or protection was concerned. We have redefined and reclassified the lesions as follows, on the basis of these early reactions after injection:

1. *Diffuse Reaction*.—With this type of reaction, the abdominal skin of the injected pig became raised as a diffuse, soggy, almost cystic swelling within the first 24 hours after injection. There was an absence of the cardinal signs of inflammation other than swelling. There was no clear-cut line of demarcation of the normal from the abnormally involved skin. In appearance, the animals were sluggish, the hair stood out and they would neither eat nor drink.

2. *Localized Reaction*.—After local injections, such pigs as showed this reaction had a localized swelling with some or all the other cardinal signs of inflammation, *i.e.*, redness, induration, etc. This inflammatory area was from 4 to 8 cm. in diameter, usually the lesion was sharply defined from normal tissue by an indurated border (probably a pyogenic membrane). The animals had local discomfort, but were active and ate well.

Either the diffuse or the localized lesion might go on to abscess formation. The diffuse lesion usually ulcerated and sloughed early, the localized abscesses late.

3. *Negative Reaction*.—There was no local reaction in these pigs.

I. Results from Compresses of Various Kinds

1. The act of irritating and shaving the abdominal wall of the pig had no effect either one way or the other on local immunity (Table I).

2. Simple water, dry compresses and saline compresses were but slightly protective (Table II).

3. Mustard plasters as compresses were effective, but the results were not so striking as with broth compresses (Table III).

4. Liebig's meat extract protected slightly better than peptone alone and about as well as broth compresses (Table IV).

5. 10 per cent gave no better protection than 1 per cent peptone (Table V).

6. Normal horse serum was found to be as efficient a protector as broth (Table VI).

Imschenetsky found that the longer applications were applied, the greater were the skin changes, a dictum to which we can subscribe. That compressing with such simple materials as normal sodium chloride solution may sometimes give reactions comparable to those obtained by broth is also shown by our experiments, but these are the exceptions rather than the rule and where such protection occurs, we would be inclined to attribute it to the pressure applied, rather than to the heat of the compress as claimed by the author.

The fact that we have obtained slight or great immunity by various procedures and materials would properly explain conflicting good results obtained with divers substances.

Guinea pigs which are "broth-protected" prior to bacterial injection live, but animals broth-protected after injection die. This parallels Gay's experience in experimental pleural infections with streptococcus.

That some materials excite the tissues to relatively great reaction, is obvious from these and our previous experiments.

CONCLUSIONS

1. Many substances besides the specific broth filtrates of Besredka can be utilized as topical applications to protect guinea pigs from the effects of massive doses of staphylococcus given subcutaneously. (Plain broth, peptone 10 per cent, peptone 1 per cent, Liebig's meat extract, mustard plaster and normal horse serum.)

2. Where such protection occurs, no matter what the stimulus is, the local skin reaction microscopically is the same as that previously described for broth compresses.

3. Many topical applications of such substances as saline, water, plain compresses, etc., may confer slight protection on an animal.

4. Specific filtrates (Besredka) confer no protection on the experimental animal if applied at the time of inoculation or thereafter.

5. The local protection described in our experiments is non-specific in its nature.

clasmatoocytes in great numbers as long as 16 days after protection was discontinued.

COMMENT

Besredka states that specific filtrates are bactericidal *in vivo* and when bacteria and specific broth filtrates are injected locally, there is no response on the part of the tissues. One, as it were, neutralizes the other. That there is no such protection is shown by our experiments. Although plain broth and specific broth filtrate are readily absorbed as such, the addition of viable staphylococcus to either and the injection of the mixture subcutaneously was followed either by death or the occurrence of a diffuse lesion in the test animal.

Imshenetsky² who worked with topical applications of Besredka's "staphylococcus broth virus," stated that his conclusions were the same as those of Freedlander and Toomey¹ except that in addition to what these authors described, he noted leucocytes in the epithelial layer, scattered and sometimes accumulated beneath the stratum corneum. He further stated that he, unlike us, was unable to find any fibroblastic proliferation. He described the effect of "staphylococcus broth virus" application, while in our paper, we pictured the effect of broth applications. Our subsequent work showed that there was no difference in skin reaction to plain broth compresses as compared to "specific staphylococcus broth compresses" (Besredka's staphylococcus antiviral), either grossly or microscopically so that Imshenetsky's error of comparison is not of any great moment. That fibroblastic proliferation existed may be noted from Fig. 11, Plate 30 of our original article¹ where such a reaction is photographed.

We also noted an increase in polymorphonuclear cells, for in our first article, we stated that "while there was a moderate number of polymorphonuclears and small mononuclear leucocytes, there was, especially in the subcutis, a marked increase in the number and size of the clasmatoocytes and elongated tissue cells." This scattered infiltration of leucocytes was not the predominating reaction and hence was not stressed. We did not perceive the distinctive reaction of the hair follicles noted by the author.

² Imshenetsky, A., *Z. Ges. exp. Med.*, 1929, 69, 113.

TABLE II

Effect of Dry Compresses, Saline Compresses and Plain Water Compresses

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Dif-fuse	Local	Neg.	Died
Protected by saline compresses	2 cc. of broth suspension	15	11/ 1/24	5	1	4	0	0
	Same as above	16	12/24/24	5	4	1	0	1
	" " "	17	1/12/25	5	5	0	0	0
	0.03 cc. bacteria in 3 cc. broth	64a	2/ 5/27	4	2	2	0	0
	Same as above	83	1/19/28	10	6	4	0	3
	" " "	84	2/ 4/28	10	7	3	0	4
Total.....				39	25	14	0	8
Protected by plain water compresses	For dosages, see corresponding experiments	15	11/ 1/24	5	5	0	0	0
		16	12/24/24	5	5	0	0	0
		17	1/12/25	5	5	0	0	0
		19	3/14/26	10	4	6	0	0
Total.....				25	19	6	0	0
Protected by dry compresses	For dosages, see corresponding experiments	17	1/12/25	5	5	0	0	0
		19	3/14/26	10	4	6	0	0
		64a	2/ 5/27	4	3	1	0	1
Total.....				19	12	7	0	1
Controls: protected by broth compresses	For dosages, see corresponding experiments	15	11/ 1/24	5	0	5	0	0
		16	12/24/24	5	0	5	0	0
		17	1/12/25	5	1	4	0	1
		64a	2/ 5/27	4	0	4	0	0
		83	1/19/28	10	1	9	0	2
		84	2/ 4/28	10	0	8	2	1
Total.....				39	2	35	2	4
Controls: not protected	For dosages, see corresponding experiments	15	11/ 1/24	5	5	0	0	0
		16	12/24/24	5	5	0	0	0
		17	1/12/25	5	5	0	0	0
		19	3/14/26	10	10	0	0	1
		64a	2/ 5/27	4	4	0	0	0
		83	1/19/28	10	8	2	0	6
		84	2/ 4/28	10	9	1	0	7
Total.....				49	46	3	0	14

TABLE I
Protective Effect of Various Simple Procedures

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Diffuse	Local	Neg.	Died
Shaved, not irritated, broth-protected	2 cc. broth culture suspension, not standardized	14	10/28/24	5	0	5	0	0
	Same as above	15	11/ 1/24	5	0	5	0	0
Total.....				10	0	10	0	0
Shaved, not irritated, not protected	For dosages, see corresponding experiments	14 15	10/28/24 11/ 1/24	5 5	5 5	0 0	0 0	1 0
Total.....				10	10	0	0	1
Shaved, irritated, not protected	For dosages, see corresponding experiments	14 15	10/28/24 11/ 1/24	5 5	4 4	1 1	0 0	0 0
Total				10	8	2	0	
Not shaved, not irritated, not protected	For dosage, see corresponding experiment	14	10/28/24	5	5	0	0	1

TABLE V

Comparative Protective Effects of Peptone Dilutions, 1 and 10 Per Cent

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Dif-fuse	Local	Neg.	Died
Protected by 1 per cent peptone compresses	0.015 cc. bacteria in 3 cc. saline	87	3/27/28	10	3	7	0	3
Protected by 10 per cent peptone compresses	Same as above	87	3/27/28	10	3	7	0	4
Protected by broth compresses	" " "	87	3/27/28	10	0	9	1	0
Controls: not protected	" " "	87	3/27/28	10	10	0	0	4

TABLE VI

Illustrating the Protective Power of Normal Horse Serum

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Dif-fuse	Local	Neg.	Died
Protected with normal horse serum compresses	0.015 cc. bacteria in 3 cc. saline	120	4/24/29	8	1	7	0	0
	Same as above	121	10/ 8/29	5	0	5	0	0
Total.....				13	1	12	0	0
Controls: unprotected	For dosages, see corresponding experiments	120	4/24/29	8	7	1	0	3
		121	10/ 8/29	5	3	2	0	0
Total.....				13	10	3	0	3

TABLE III
Protective Effect of Mustard Plaster Compresses

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Dif-fuse	Local	Neg.	Died
Mustard plaster compresses	0.015 cc. bacteria in 3 cc. saline	69	2/17/27	8	6	2	0	2
	Same as above	71	3/31/27	8	2	6	0	1
Total.....				16	8	8	0	3
Protected by broth compresses	For dosages, see corresponding experiments	69	2/17/27	8	1	7	0	0
		71	3/31/27	8	1	7	0	1
Total.....				16	2	14	0	1
Controls: no protection	For dosages, see corresponding experiments	69	2/17/27	8	8	0	0	4
		71	3/31/27	8	8	0	0	1
Total.....				16	16	0	0	5

TABLE IV
Protective Effect of Peptone Compresses

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Dif-fuse	Local	Neg.	Died
Protected by 1 per cent peptone compresses	0.015 cc. bacteria in 3 cc. saline	85	2/29/28	10	5	5	0	2
Protected by Liebig's meat extract, 1 per cent	Same as above	85	2/29/28	10	1	3	6	0
Controls: broth-protected	" " "	85	2/29/28	10	0	4	6	0
Controls: not protected	" " "	85	2/29/28	10	10	0	0	4

TABLE VIII

Illustrating the Length of Time Protection Lasts after Broth Compresses

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Dif-fuse	Local	Neg.	Died
Broth compresses, protected pigs, injected 24 hrs. after protection ceased	0.015 cc. bacteria in 3 cc. saline	76	6/ 7/28	6	0	2	4	0
Controls: not pro-protected				6	6	0	0	2
72 hrs. after protec-tion ceased	Same as above	76	6/7 /28	3	0	2	1	0
Controls: not pro-protected				3	3	0	0	1
120 hours after broth-protection ceased	" " "	76	6/ 7/28	3	0	3	0	0
Controls: not pro-protected				3	3	0	0	1
168 hours after broth-protection ceased	" " "	76	6/ 7/28	3	1	2	0	1
Controls: not pro-protected				3	3	0	0	0
216 hours after broth-protection ceased	" " "	76	6/ 7/28	2	2	0	0	2
Controls: not pro-protected				2	2	0	0	2

TABLE VII

Illustrating the Neutralizing Effect of Specific Broth Filtrates (Besredka)

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Diffuse	Local	Neg.	Died
Group A: protected by broth compresses; positive controls	0.015 cc. bacteria in 3 cc. saline	77	7/11/27	10	1	5	4	0
Group B: controls; not protected	Same as above	77	7/11/27	10	6	2	2	1
Group C: not protected	0.015 cc. bacteria in 3 cc. specific broth	77	7/11/27	10	10	0	0	4
Group D: not protected	0.015 cc. bacteria in 3 cc. plain broth	77	7/11/27	10	10	0	0	5
Group E: injected to time absorption	3 cc. plain broth	77	7/11/27	10	0	0	10	0
Group F: injected to time absorption	3 cc. specific broth	77	7/11/27	10	0	0	10	0

TABLE IX

Effect of Broth Protection Applied after Bacterial Injection

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Diffuse	Local	Neg.	Died
Controls: protected by broth compresses before injection	0.015 cc. bacteria in 3 cc. saline	74	5/ 5/27	8	0	8	0	2
	Same as above	75	5/24/27	8	1	7	0	0
	" " "	89	5/21/28	12	0	7	5	0
Total.....				28	1	22	5	2
Controls: not protected before injection	For dosages, see corresponding experiments	74	5/ 5/27	8	8	0	0	8
		75	5/24/27	8	8	0	0	1
		89	5/21/28	12	7	5	0	0
Total.....				28	23	5	0	9
Animals not protected before, but after injection by applying broth compresses to the injected area	For dosages, see corresponding experiments	74	5/ 5/27	8	8	0	0	8
		75	5/24/27	8	5	0	3	6
		89	5/21/28	16	11	5	0	14
Total.....				32	24	5	3	28

TABLE X

Reinjection Experiments to Illustrate the Length of Time Protection Lasts

Procedure	Dosage	No. of exp.	Date injected	No. pigs used	Reaction			
					Diffuse	Local	Neg.	Died
These animals had been previously injected and had recovered. Injected 30 days previous	0.015 cc. bacteria in 3 cc. saline	88	5/ 4/28	10	5	5	0	1
Injection 21-40 days previous	Same as above	33	9/ 5/28	9	0	9	0	0
Controls: not protected	" " "	88	5/ 4/28	10	10	0	0	6

20 minutes and the supernatant fluid was pipetted off for use. The menstruum used throughout was a fluid composed of one-third normal rabbit serum, and two-thirds Tyrode's solution.

At the end of each experiment the different suspensions (trituated in a sterile mortar without sand if tissue was present) were tested for the presence of vaccine virus by inoculating 0.25 cc. into the shaved skin of a rabbit. Stained films of each preparation were examined and all those showing bacterial contamination were discarded.

In quantitative determinations, serial dilutions were made in Locke's solution in multiples of ten, and 0.25 cc. of each dilution was inoculated into the shaved skin of a rabbit.

Each experiment was controlled by adding virus to normal tissue in a mixture of serum and Tyrode's solution according to the technique of Maitland and Maitland (2), and incubating in test tubes and in Carrel flasks. The virus invariably survived under these conditions, and the reactions resulting from inoculation of the virus in the Carrel flasks were always greater than from that in the test tubes. This would be expected from the observations of Maitland and Laing (3) on the necessity of free access of air for the increase of the virus, since the ratio of surface to volume in Carrel flasks is greater than in test tubes.

Attempts at Passage in Series

Experiment 1.—Normal minced kidney tissue was placed inside the collodion sac of a dialyzing apparatus. In the outer chamber, vaccine virus was placed with kidney cells that had been killed by repeated freezing and thawing. This fluid containing virus had first been titrated by inoculating serial dilutions into the skin of a rabbit.

The dialyzing apparatus was opened after 4 days incubation at 37°C. A portion of the fluid in the outer chamber, which contained virus, was diluted 1:10 in a fresh suspension of killed cells and placed in the outer chamber of another dialyzing apparatus, with a fresh suspension of normal minced kidney tissue in the inner chamber. These passages were always made in duplicate. The virus removed from the outer chamber was titrated by intracutaneous inoculation of serial dilutions. The suspension of living cells from the inner chamber was always tested by intradermal inoculation to control the impenetrability of the membrane to vaccine virus.

This procedure was repeated with each passage.

The inoculated rabbits were observed daily, and the highest dilution showing a vaccinal reaction was considered to be the titer of the virus. By recording the highest active dilution it was possible to construct a curve showing the variation in concentration of the virus as the experiment progressed.

The results of two typical experiments are shown in Fig. 1. One line shows the curve that would be expected if survival were complete

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FURTHER OBSERVATIONS ON THE SURVIVAL OF VACCINE VIRUS SEPARATED FROM LIVING HOST CELLS BY COLLODION MEMBRANES

By RALPH S. MUCKENFUSS, M.D.

(From the Department of Internal Medicine, Washington University, St. Louis)

(Received for publication, November 28, 1930)

In a previous communication (1) it was reported that vaccine virus diluted in a solution of one part serum and two parts Tyrode's solution survived incubation for 4 days at 37°C. if separated by a semipermeable membrane from a suspension of minced kidney tissue in a similar mixture of serum and Tyrode's solution. The survival of the virus, however, was not complete. If kidney cells that had been killed by repeated freezing and thawing were added to the vaccine virus under these conditions, the survival as determined by the intensity of the skin reaction in rabbits was greater, and seemed to be almost complete. The possibility was considered that the virus might increase under these conditions and this was the first point studied in the present series of experiments.

Methods and Materials

The technique was detailed in the previous report (1). Briefly, the apparatus consists of two tubes, one of which can be inserted into the other. A shoulder is made on the smaller tube and ground to fit the top of the larger tube, making a perfect joint. The lower end of the inner tube is left open, and a collodion sac is attached to it, thus making the assembled apparatus consist of two chambers separated by a collodion membrane. The collodion sacs were arbitrarily standardized by measuring the amount of water that would pass through in 3 minutes under a pressure of 1 m. of water. The membranes used in this series of experiments varied in permeability from 0.65 cc. to 1.09 cc. of water passing through the membrane in 3 minutes, with an average permeability of about 0.75 cc.

The neurovaccine of Levaditi, prepared by testicular inoculation was used exclusively. The testicles of rabbits were removed on the 4th day after inoculation and, after trituration with sand in a sterile mortar, about 10 cc. of Locke's solution was added. The testicular emulsion was centrifuged at high speed for

variations of Duran-Reynals (4) on the influence of tissue extracts on vaccinal reactions, it seemed desirable to ascertain if the tissue played any part in the greater intensity of the reactions that had been previously observed. The work of Duran-Reynals was done with fresh tissue. Because of the 4 day period of incubation employed in this study, the action of fresh tissue was compared with that of tissue which had been incubated for 4 days. The action of normal cells was also compared with that of cells that had been killed by repeated freezing and thawing.

Experiment 2.—Two suspensions of minced kidney tissue similar to those previously used were prepared. Normal kidney tissue was used in one tube, and cells that had been killed by repeated freezing and thawing were used in the other. These tubes were incubated for 4 days. Two similar preparations were then made from fresh materials. Vaccine virus was then added to all of these tubes and also to a tube of serum and Tyrode's solution containing no tissue. After standing for a few minutes the emulsions containing tissue were triturated, and 0.25 cc. from each tube was inoculated into the shaved skin of a rabbit.

The rabbit was observed daily for 4 days and the reactions recorded.

While there was some increase in the intensity of the reactions in the presence of tissue, the increase was very slight, and there was no difference in the effects of the different tissue preparations.

This experiment was conducted with freshly prepared virus, and it seemed desirable to repeat the experiment using virus and cells that had been handled as nearly as possible in the same manner as had been done in experiments using the dialyzing apparatus.

Experiment 3.—In one dialyzing apparatus normal cells were placed in the inner chamber and vaccine virus in the outer chamber. In another, normal cells were placed in the inner chamber and a suspension of killed cells in the outer chamber. These, along with a suspension of normal cells in a test tube were incubated for 4 days. After incubation the virus from the outer chamber was removed and divided into three portions. To one portion an equal quantity of Locke's solution was added; to the second an equal quantity of the dead cells from the outer chamber of the second dialyzing apparatus was added; and to the third an equal quantity of the incubated suspension of normal cells. The suspensions containing cells were triturated and intracutaneous inoculations were made from each mixture.

Table I, which is typical of several such experiments, shows that the reaction was intensified by the addition of cells. In this experiment

and the concentration had diminished directly in proportion to dilution. The other two lines, recording the observations in two experiments, show that survival was not complete. With each passage there was a decrease in concentration, and the quantity of virus demonstrable

PERSISTENCE OF VACCINE VIRUS IN DIALYZING APPARATUS

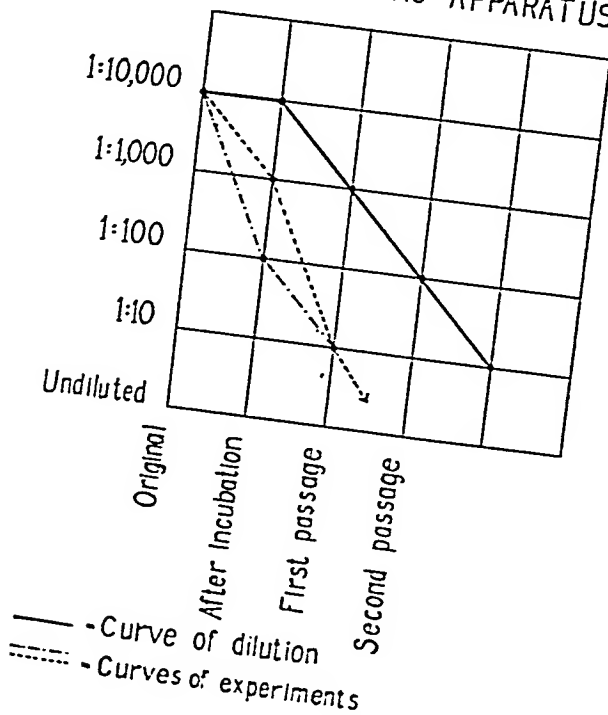


FIG. 1

at the end of each period of incubation was less than had been placed in the dialyzing apparatus.

It seems probable, therefore, that the intense reactions observed in the experiments previously reported represented the survival of a considerable part, but not all, of the virus.

The Effect of Tissue on the Intensity of the Reaction

In the original report it was shown that the reaction was more intense if virus was incubated with dead cells. In view of the obser-

gestion is further borne out by one experiment recorded in Table IV, in which no survival of the virus alone could be detected, although a definite reaction followed inoculation of virus that had been incubated with dead cells.

TABLE III

Container used for incubation	Vaccine virus	Vaccine virus plus dead cells	Vaccine virus plus extract of dead cells
Test tube.....	—	—	—
Dialyzing apparatus.....	—	+	?
Test tube.....	—	±	±
Dialyzing apparatus.....	++	+++	+

TABLE IV

Container used for incubation	Normal kidney plus vaccine virus (Maitland culture)	Vaccine virus		Vaccine virus plus dead cells		Vaccine virus plus cysteine hydrochloride		Vaccine virus plus dead cells plus cysteine hydrochloride	
Test tube.....	+++	—	—	—	—	—	—	—	?
Carrel flask.....	+++±	—	—	—	—	—	—	—	—
Dialyzing apparatus..		+		++		++	++	++	++
Test tube.....	+	—	—	+		?	?	±	±
Carrel flask.....	++++	—	—	+		?	?	±	±
Dialyzing apparatus..		+±	++	++±	++	+	+±	++	+++
Test tube.....	+	?	—	±		+±	?	±	?
Carrel flask.....	+++	—	—	—		—	—	±	±
Dialyzing apparatus..		+±	+	+++	+++	+±	+±		+++
Test tube.....	+	—	—	—		—	—	—	—
Carrel flask.....	++	—	—	±		±	—	+±	+±
Dialyzing apparatus..		±	±	++	++	+	±	+++	+++

The method used is inaccurate at best, and when virus is mixed with tissue, a special source of error must be considered. Even after several minutes trituration, some gross fragments of tissue were still present, and it is possible that these either adsorbed or took up in other ways a considerable amount of virus, making accurate dilution impossible.

the reagents were the same as in the inoculations from the dialyzing apparatus in previous experiments, but the virus and tissue were diluted to half their original concentration in placing them together.

The results of these experiments cannot be compared quantitatively with those of Duran-Reynals, as the quantities that he employed differ from those used in this study.

The Effect of Dead Cells on Survival

The action of kidney tissue in enhancing the skin reaction to vaccine virus made necessary a quantitative study of the survival of virus alone, as contrasted with the survival of virus to which killed cells had been added.

TABLE I

Control (virus plus Locke's solution).....	++
Virus plus frozen and thawed kidney.....	+++
Virus plus normal minced kidney	++++±

TABLE II

Date	Virus plus dead cells	Virus without dead cells
Nov. 25, 1929	10^{-4}	
June 28, 1930	10^{-2}	10^{-2}
	10^{-3}	10^{-3}
	10^{-2}	10^{-2}
		10^{-2}

Experiment 4.—The same arrangement of the dialyzing apparatus, with living cells in the inner chamber, was employed. Virus alone was placed in the outer chamber of some, while virus with dead cells was placed in the outer chamber of others. After 4 days incubation the different virus preparations were titrated in the manner previously described.

The results of four such parallel titrations are recorded in Table II. In two instances the titer was higher when dead cells were present; in the other two the same titer was obtained.

While the difference is not striking, the method used can measure concentration only in multiples of ten, and the results suggest an increased survival of virus in the presence of dead cells. This sug-

Berkefeld N candle. The dialyzing apparatus was used as before, and in addition, the solution of cysteine hydrochloride was added to some of the virus preparations in the outer chambers so that the final concentration was 0.1 per cent or 0.15 per cent. After incubation for 4 days intracutaneous inoculations were made.

The results recorded in Table IV show that, while the skin reactions in a few instances were somewhat greater when cysteine hydrochloride had been added, this was not uniformly the case, and the effect was not great in any instance.

COMMENT

The results strongly suggest that the survival of vaccine virus made possible by the presence of live cells on the opposite side of a collodion membrane is further increased by admixing dead cells with the virus. It might be urged that the more intense skin reactions are due entirely to the effect of kidney tissue in enhancing the local reaction (Reynals factor). However, titration by serial dilution suggests an increased survival, and in one experiment survival occurred in the presence of dead cells while failing to occur in their absence. Extracts of dead kidney cells had no enhancing effect under the conditions of these experiments.

The reason for the survival of virus when incubated on the opposite side of a collodion membrane from a suspension of normal kidney tissue is uncertain. Two possibilities, however, suggest themselves. One is that some factor necessary for survival diffuses through the membrane from the living cells to the virus; the other is that the living cells utilize some substance, possibly oxygen, which if present in sufficient concentration would result in the inactivation of the virus.

SUMMARY

The survival of vaccine virus when incubated on the opposite side of a collodion membrane from a suspension of fresh minced rabbit kidney was not complete in these experiments, and passage in series was not successful. The degree of survival seemed somewhat greater if dead cells, killed by repeated freezing and thawing, were added to the virus during incubation, although the tissue was able to increase the intensity of the skin reactions. Extracts of dead kidney cells did not increase the degree of survival, as determined by the intensity of

The Effect of Tissue Extracts on the Survival of Virus

It seemed desirable to ascertain if the presence of the dead cells themselves was necessary for the increased intensity of the skin reactions. To study this question, extracts of the dead cells were prepared, and the action of the extracts was compared with the action of the extracted cells.

Experiment 5.—Minced kidney tissue that had been repeatedly frozen and thawed was added to Tyrode's solution in the proportion of 0.66 cc. of tissue to 12 cc. of Tyrode's solution. After thorough mixing, the suspension was allowed to stand for a few minutes and then centrifuged. The supernatant fluid was pipetted off, and serum and vaccine virus were added to it in the proportions previously described. The cells were resuspended in Tyrode's solution, and serum and vaccine virus added in the same manner.

These suspensions were incubated for 4 days on the opposite side of collodion sacs from live cells and then were tested by intracutaneous inoculation into rabbits.

The results were somewhat variable, but there was never any appreciable enhancement of the reaction as a result of the addition of the extracts, and in most instances the size of the reactions was diminished. In Table III the results of two typical experiments are recorded. Although the extracts were inactive, the extracted cells were still capable of causing an increased skin reaction. It is interesting to note that in the first experiment recorded in the table, the vaccine virus alone did not survive in the outer chamber of the dialyzing apparatus, although the virus with dead cells showed definite survival. This is the only experiment in which virus has not survived under the experimental conditions described.

The Effect of Cysteine

The observations of Mueller (5), Zinsser and Tang (6) and of Long and Olitsky (7) on the usefulness of cysteine in the preservation of viruses, together with the observations of Dubos (8) on its rôle in culture media used for the cultivation of pneumococci, made it desirable to ascertain if this substance could have any effect on the survival of vaccine virus under the experimental conditions used in this study.

Experiment 6.—A 10 per cent solution of cysteine hydrochloride (Pfanstiehl) in phosphate buffer at pH 7.6 was prepared and sterilized by filtration through a

the skin reaction. No significant or constant increase in the intensity of the skin reactions resulted from the addition of cysteine hydrochloride to the virus in the dialyzing apparatus.

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has been claimed that testicle or kidney pulp makes an excellent medium for the multiplication of viruses *in vitro* (12, 13), but later work has shown that the methods of preparation of the pulps employed did not eliminate living cells (14). The activity of testicle extract *in vivo* is demonstrated by its stimulating action on the healing of chronic refractory ulcers (15, 16). The lymphoid tissue, which seems to have the opposite effect from testicle extract on the virus infections is known to exert an inhibitory influence on cell growth (17).

Previous work (2) from this laboratory has shown that if testicle extract is injected intracutaneously and vaccine virus intravenously, the virus infection will be sharply localized to the skin area in which testicle extract has been injected. The phenomenon will be referred to later in the present paper, but it may be said in this connection that localization of various intravenously injected dyestuffs, notably methylene blue and trypan blue, also occurs in several pathological processes, as *e.g.* pneumonia, encephalitis, experimental tuberculosis and inflammation brought about by thermal agents, mustard oil, aleuronat, turpentine, etc. (Menkin, Friedheim and others (18, 19)).

An experimental observation has provided a clue for the investigation of the mode of action of the Reynals factor. The wheal resulting from the intracutaneous injection of mixtures containing testicle extract disappeared much more rapidly than those caused by mixtures not containing the extract. This suggested that the testicle extract may produce its effect by influencing tissue permeability. The present study was undertaken to test this possibility.

Methods and Materials

Organ extracts were prepared by grinding the tissue with sand and its own volume of Ringer's solution. The pulp was centrifuged and the supernatant fluid used.

In order to study the rate of spread after intracutaneous injection of testicle extract, or any other organ extract, it was essential to render the injected mass visible through the superficial epidermal layers.

For this purpose we employed in the preliminary tests a mixture of equal parts of iron ammonium citrate and potassium ferrocyanide, each in a 0.5 per cent aqueous solution. Injections of 0.5 cc. of the mixture plus an equal volume of rat or rabbit testicle extract, or of Ringer's solution as control, were made in different areas of the shaved skin of the rabbit. Thereafter, at intervals of 10 minutes, small pieces of skin were removed and fixed in a 10 per cent formalin solution which con-

THE INFLUENCE OF TESTICLE EXTRACT ON THE INTRA- DERMAL SPREAD OF INJECTED FLUIDS AND PARTICLES*

BY D. C. HOFFMAN, M.D. AND F. DURAN-REYNALS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 18

(Received for publication, November 13, 1930)

It has been shown by one of us (1, 2, 3) that extracts of certain organs of the body have an enhancing action on the development of vaccine virus and staphylococcus infections and that all organs studied can be placed in three groups according to the effect of their extracts. Group I comprises the testicle, kidney, brain and skin, the extracts of which always enhance the infections. Group II contains the adrenal, retina, ovary and the entire embryo, the extracts of which do not modify the infections. Group III includes blood, spleen, lymph nodes and bone marrow, the extracts of which tend to lessen or in some instances even to suppress the infections.

Of all the organs of the first group the testicle is by far the most active. The enhancing power of its extracts has been shown to extend to other infections, besides those above mentioned as demonstrated by Hoffman (4) with the viruses of herpes, vesicular stomatitis and Borna disease, by Thompson (5) in one observation with poliomyelitis virus and Pijoan (6) with 20 different bacteria. The active principle responsible for the enhancing effect has been referred to by Ledingham and Barratt (7) as the Reynals factor and for convenience the term will be used in this paper.

The nature of the action of testicle extract has not been understood, although there are observations in the literature which have a bearing on the interpretation of the enhancement phenomenon. Thus the stimulation of cell growth *in vitro* by testicle as well as by other organ or tissue extracts has been well established (8, 9, 10, 11). It

* A preliminary report of this work appeared in *Science*, 1930, 72, 595.

Influence of Testicle Extract on the Spreading of India Ink

Eleven experiments were performed with rat and rabbit testicle extract plus India ink. As shown in Chart 1, the mixtures of India

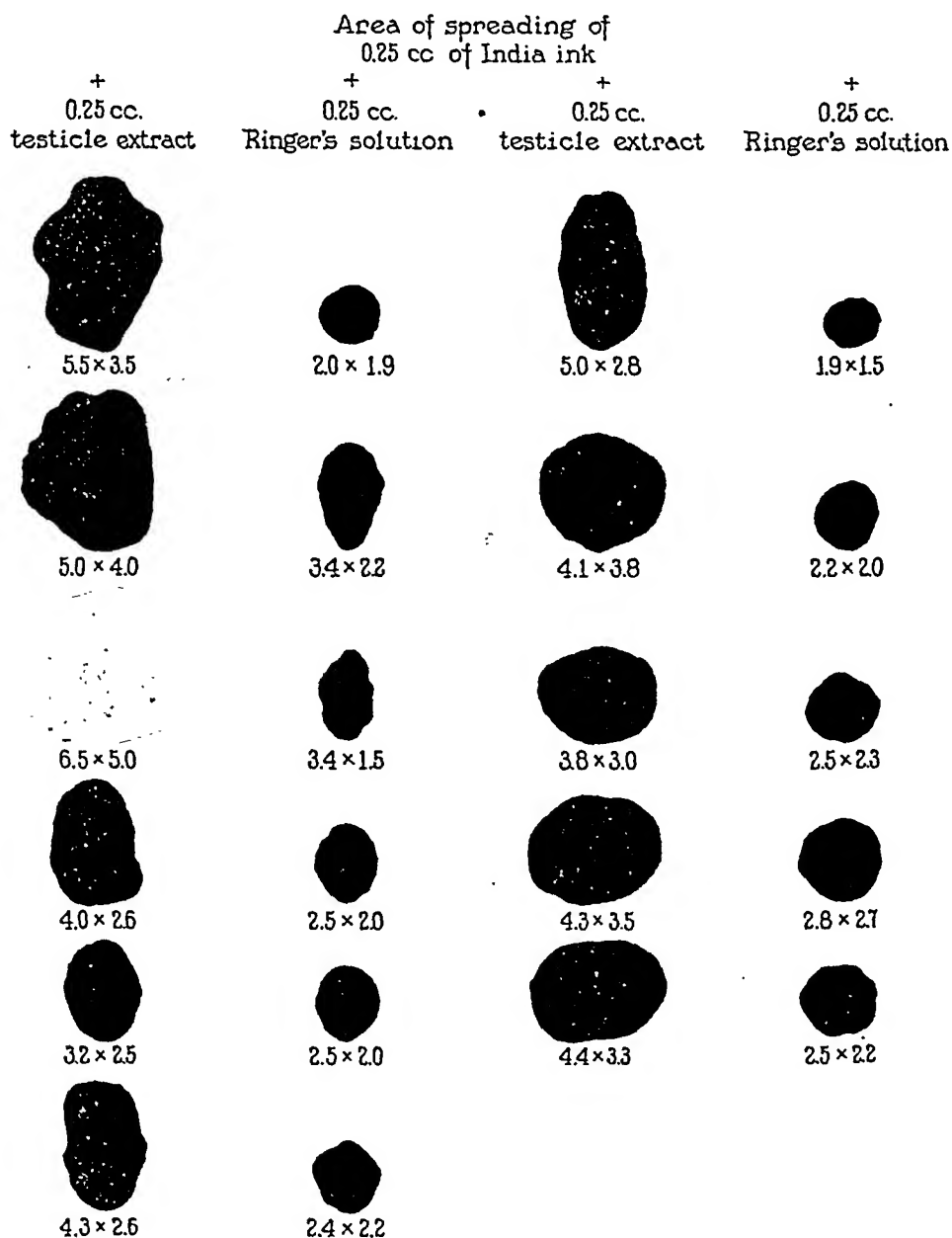


CHART 1

tained 5 per cent HCl.* This precipitated the Prussian blue. Microscopic sections were prepared in the customary manner and counterstained very lightly with hematoxylin. In the later experiments recourse was had to India ink diluted 1:2 with Ringer's solution for with this material the rate of spread could be more accurately followed.

The diluted ink in quantities of 0.25 cc. was mixed immediately before injection with an equal amount of organ extract or with Ringer's solution as control, and injected into a number of different spots in the shaved skin of the same rabbit. 1 hour later the areas in which the ink had spread were measured and recorded. They could still be discerned after 24 hours or even later, and indeed a gray spot was well defined for weeks after, but no essential increase in the area involved occurred after 1 hour. In several cases the rabbit was killed at this time and the various injected areas were excised and fixed for histological examination. At first ordinary staining with eosin and methylene blue was used. Later we found that diffuse coloration with a 1:100 picric acid solution gave a background on which the ink particles contrasted sharply.

Influence of Testicle Extract on the Spreading of Iron Ammonium Citrate and Potassium Ferrocyanide

Five experiments were performed as described. In each the wheal caused by the injected mixture containing testicle extract flattened out and disappeared very promptly, and after a few minutes a blue discoloration of the skin alone remained. The wheal caused by the injection of the dye alone persisted in many instances for more than 1 hour. Microscopic examination of it showed a rather diffuse blue color throughout the intracellular spaces. There was much edema and at the edge of the section the Prussian blue had been precipitated in large quantities. In only a very few instances was there any suggestion of the dye having penetrated cells. On the other hand, the sections taken from the areas which had received the mixture with testicle extract showed a much fainter blue color, and most of them had granules or masses of the dye within the cells of the connective tissue (see Fig. 1). Only in one instance were the blue particles seen within epithelial cells, but not infrequently endothelial cells of the blood vessels were full of them. Sections of skin removed 30 to 40 minutes after the injection showed the greatest amount of Prussian blue within the cells. It was also noted that the dye very commonly was seen adhering to the cell membranes.

* All animals subjected to operation were etherized.

but in the others proved inactive in this respect. The comparative results are better seen in Chart 3 where the averages of the areas of spreading induced by different organ extracts are shown.

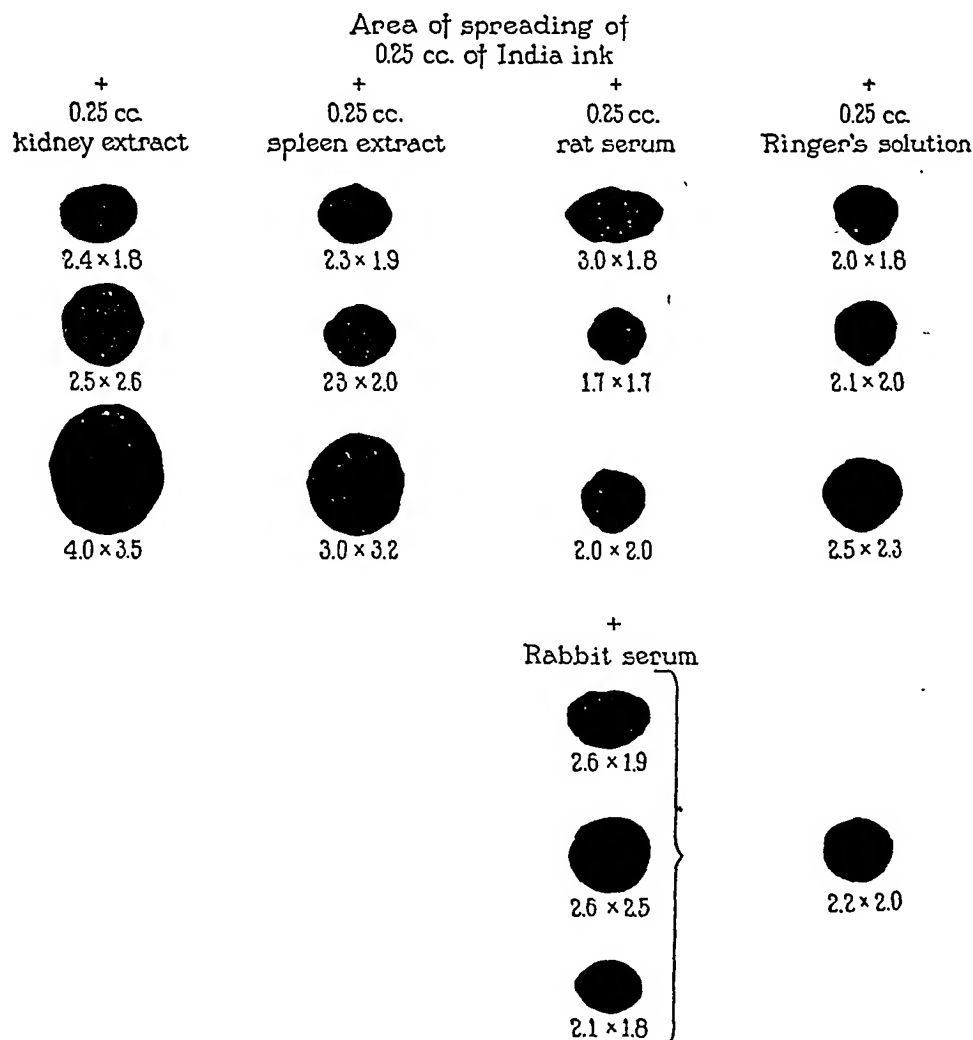


CHART 2

Influence of Testicle Extract on the Localization of Methylene Blue

As already remarked, methylene and trypan blue injected intravenously tend to come out especially into areas affected by pathological processes. The fact has a possible relation to the localization of vaccine virus in the skin area which has received an injection of testicle ex-

ink plus testicle extract had spread over a much larger area than was the case with the control material of India ink in Ringer's solution.

Sections were taken of both areas. In those receiving India ink plus testicle extract the ink particles were widely distributed, gradually thinning out towards the edges, and they could be seen far from the point of inoculation. The ink particles outlined the contours of the cells, sometimes indeed appearing to be within the cell protoplasm, but this can not be definitely affirmed without more refined histological methods (see Fig. 2). In the control areas the spreading of the ink stopped suddenly at a short distance from the injected locus and the particles were in general independent of the cells. In both cases, the ink spread only through the connective tissue spaces, the Malphigian layers, muscle bundles and hair follicles being free from it. In only rare cases, and where testicle extract had been injected, could some particles be seen between the muscle bundles, but none were observed to have penetrated the muscle sheath.*

Influence of Kidney and Spleen Extracts and Blood Serum on the Spreading of India Ink

The foregoing experiments reveal that the Reynals factor increases the spread through the tissues of both soluble and particulated matter. This property may be responsible at least in part for the enhancement of infections. The parallelism between the spreading and the enhancement phenomena was next tested by a study of the influence of other organ extracts. The results of the experiments are shown in Chart 2.

One can note in Chart 2 that extracts of organs belonging to Group I, the enhancing group, have an effect similar to that of testicle extract, but kidney extracts cause a less degree of spread and the results are not so consistent. Rat or rabbit blood serum, which has an inhibiting influence on infections, does not increase the spreading of India ink, but indeed sometimes slightly interferes with it. Spleen extract, which is also an inhibitor, in one test gave a slightly increased spread,

* In this connection it is of interest to note that testicle extract does not render healthy conjunctiva more permeable to methylene blue, when the mixture of dye and extract is instilled into the eye; nor is the wall of the digestive tract rendered more permeable to proteins, for animals fail to become sensitized when fed foreign proteins with testicle extract.

of 1 per cent methylene blue was injected intravenously. Shortly after the dye injection, the treated skin areas turned faintly blue, but the color soon faded. When 20 cc. of the dye was injected the whole shaved skin together with the nose and lips of the animal exhibited a faint bluish color which was more marked, however, in the injected cutaneous areas. In some instances, after a few minutes, these areas became more blanched than the surrounding skin, owing probably to a reduction of the dye by the living cells. The basis for this

TABLE I

No. of guinea pigs	Amount tetanus toxin	Amount rat testicle extract	Ringer's solution	Resultant local tetanus
	cc.	cc.	cc.	
1	2.64	0.5	—	++++ (Died in 5 days)
2	1.98	0.5	—	+++
3	1.32	0.5	—	+++
4	1.32	0.5	—	+++
5	0.66	0.5	—	+++
6	0.33	0.5	—	+++
7	2.64	—	0.5	+ ++++ (Died in 4 days)
8	1.98	—	0.5	++++ (Died in 6 days)
9	1.32	—	0.5	+++
10	1.32	—	0.5	+++ (Died in 24 hours)
11	0.66	—	0.5	+++
12	0.33	—	0.5	+++

supposition lies in the fact that when the blanched area of skin was removed and immersed in a solution of a hydrogen peroxide it showed a reappearance of the blue color. Control spots injected with serum or spleen extract did not show any greater coloration than the surrounding skin.

The Effect of the Reynals Factor on Toxins and Enzymes

We next attempted to determine the effect *in vivo* of the Reynals factor on toxins and enzymes.

tract. For the next experiment dyes were injected intravenously into rabbits having testicle extract in the shaved skin.

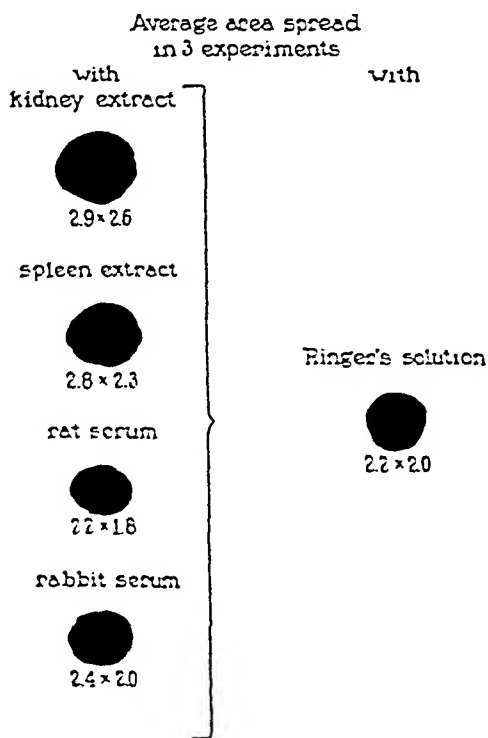
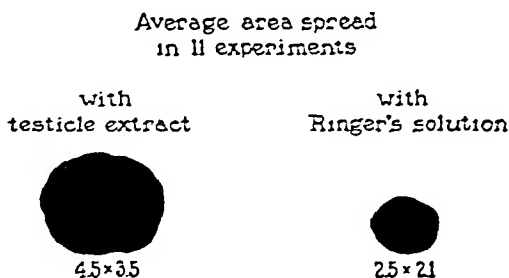


CHART 3

Four rabbits were injected intracutaneously with 1 cc. of testicle extract in two separate areas and immediately thereafter 10 or 20 cc.

fact that their spreading, as shown by the rate of disappearance of the initial bleb, was affected.

The Influence of Heat on the Spreading Power of the Reynals Factor

We have previously determined that the enhancing influence of testicle extract on vaccine virus is lost when the extract is heated to 60° for half an hour. A test was not made of whether heating would interfere with the spreading of India ink. A rabbit was injected in two areas with each of the following mixtures: India ink plus its volume of fresh testicle extract, India ink with the extract which had been heated to 60°C., the ink with extract heated to 80°C. for 30 minutes, and as control the ink diluted with Ringer's solution. A spreading over four times the area of the control resulted from the use of fresh testicle extract, whereas mixtures containing the heated extracts resulted in no greater spread than in the case of the control. Evidently heating to 60° destroys not only the power to enhance a virus but that to increase the spread of India ink.

Relation of the Reynals Factor to the Spermatogenic Cells of the Testes

Some earlier observations have shown that only a very feeble enhancement of infections if any results from extracts prepared from cryptorchic testicles in which the spermatogenic function is practically absent, but which are known to be rich in interstitial tissue. Later experiments, comprising four tests, have revealed that extracts of the epididymis from rat or rabbit have as great an enhancing effect as testicle extract. In one test rabbit sperm as such was found to contain a considerable amount of the enhancing factor. We believe that this is evidence in favor of correlating the Reynals factor in the testicle with the sperm and with actively dividing spermatogenic cells. That this factor is not the same as the male sex hormone is shown by the absence of any virus enhancing power in lipid testicle fractions highly active in provoking the growth of the comb and wattles in castrated roosters.* Work is now under way by one of us to ascertain the nature and location in the tissues of the enhancing material.

* We wish to express our indebtedness to Dr. Koch of the University of Chicago for supplying us with his highly active lipid preparation.

Trypsin.—Two rabbits were injected into the shaved skin with 1.5 cc. of a mixture made by diluting Fairchild-Foster's trypsin to 1:100 and adding to it the same volume of rabbit testicle extract. Two other control areas received the same amount of the trypsin solution diluted with its own volume of Ringer's solution. The mixtures of trypsin and testicle extract spread immediately after the injection through a much larger area than the control material as shown by the disappearance of the wheal. The resultant lesion, however, was of the same extent in each of the four areas.

Tetanus Toxin.—In two experiments involving 12 guinea pigs, weighing from 200 to 300 gm., tetanus toxin was employed with an M.L.D. of 0.000066 cc. This toxin was diluted to 1:10,000. It was then injected under the skin in the inner part of the hind leg in the amounts shown in Table I, together with rat testicle

TABLE II

Experiment	<i>B. coli</i> toxin	Testicle extract	Ringer's solution	Size of resultant lesion
	cc.	cc.	cc.	cm.
1	0.5	0.5	—	5 x 3.5
	0.5	—	0.5	5 x 4
	1.0	1.0	—	5.5 x 3.8
	1.0	—	1.0	7.3 x 3.7
2	0.5	0.5	—	7.2 x 4.5
	0.5	—	0.5	8 x 3
3	0.5	0.5	—	6 x 6
	0.5	—	0.5	6 x 5.5
	1.0	1.0	—	9 x 6
	1.0	—	1.0	5.5 x 4

extract or Ringer's solution as a control. As Table I shows, not only was the effect of the toxin unenhanced but to a certain degree its action was inhibited.

B. coli Endotoxin.—A two weeks' old culture of *B. coli* in broth was filtered through a Berkefeld V filter and the filtrate was injected into the shaved skin of 3 rabbits, together with rat testicle extract. Control mixtures of the endotoxins with Ringer's solution were also injected. This endotoxin brought about in from 12 to 24 hours a reddish, elevated lesion which had clear-cut edges, easily measured, and which disappeared in from 3 to 4 days. That testicle extract did not increase the size of the lesion is shown in Table II.

Thus the three agents, trypsin, tetanus toxin and *B. coli* endotoxin, which were active by themselves but which are inanimate substances, were not enhanced in their activity by the Reynals factor despite the

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EXPLANATION OF PLATE 18

FIG. 1. Subcutaneous area of skin. Testicle extract plus Prussian blue with light hemotoxylin counterstain. Showing cells which contain numerous granules of the blue as indicated by small arrows.

FIG. 2. Subcutaneous area of skin. Same magnification. India ink plus testicle extract with picric acid counterstain. Showing much the same phenomenon as Fig. 1, but in more marked form.

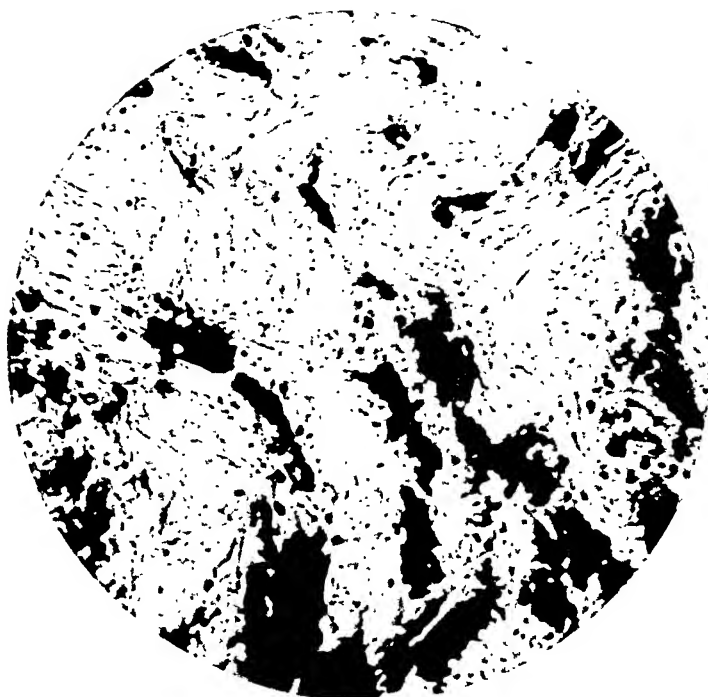
DISCUSSION AND CONCLUSIONS

The experiments in this paper show that testicle extract causes India ink particles and those of Prussian blue to spread much more extensively through the intercellular spaces than similar suspensions made with Ringer's solution. Methylene blue inoculated intravenously localizes more extensively in areas previously injected with testicle extracts than in control areas receiving injections of tissue extracts without enhancing power. Kidney extracts have this property to a less degree, whereas spleen extracts and blood serum are devoid of it. The spreading power of extracts is destroyed by heating at 60°C. for 30 minutes, as is also the power to enhance infections. The precise mode of action of the Reynals factor is not known, but the results of the experiments here presented suggest that it may depend at least in part on the property whereby testicle extract increases the spread of injected material and alters the permeability of tissue cells. It is not inconceivable that changes in permeability facilitate the passage of vaccine virus through the endothelial cells of the blood and lymph vessels, and lead to the generalized vaccinia which is of frequent occurrence in the reported results (20).

It has been shown that fluids and suspensions of inert particles are spread by the extract. *B. letanicus* and *B. coli* exotoxins and trypsin were not enhanced at all in their action despite the fact that they were spread through a more extensive area in the tissues. Viruses, on the other hand, are markedly influenced and in this respect resemble bacteria, not toxins and enzymes. It appears probable that a definite capacity for multiplication on the part of an injected substance is required if its pathogenic effects are to be enhanced. It may be concluded tentatively that the enhancing power of the testicle extract may depend on that property which not only spreads the injected material through a larger area but renders the tissue cells more easily penetrable by the agents.

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H. C. and D. C. (Fig. 18) (T. 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100)

EXPERIMENTAL

The experiments carried out were of three types: simple observations on the inactivation of poliomyelitis virus by aluminum hydroxide; studies of the effect of the pH of the mixture on this inactivating power; and determinations of the value of the inactivated virus in the production of immunity. Intracerebral inoculations of the aluminum suspension alone were without pathological effect.

Table I presents an experiment of the first kind. The aluminum hydroxide was found consistently to inactivate virus filtrate when

TABLE I
Inactivation of Poliomyelitis Virus by Aluminum Hydroxide

No.	Date	Material tested	Amount	Virus filtrate	Result
			cc.	cc.	
1	10/30	Aluminum hydroxide	0.05	0.05	No symptoms
2	10/30	Control	—	0.05	Typical poliomyelitis, 7 days
3	11/18	Aluminum hydroxide	0.1	0.2	No symptoms
4	11/18	Aluminum hydroxide	0.02	0.2	Typical poliomyelitis, 5 days
5	11/18	Control	—	0.2	Typical poliomyelitis, 14 days

equal volumes were employed. When one part of adsorbent was used with two parts of virus the same effect was observed but proportions of 1:10 failed to inactivate. Control animals inoculated with similar volumes of untreated virus contracted typical poliomyelitis.

To study the effect of the pH, a mixture of equal parts of virus and adsorbent was prepared and divided into two parts. By the use of a suitable phosphate buffer the pH of one was adjusted to 5.5 and that of the second to 8.8. Both were centrifuged at 3000 R.P.M. for 20 minutes and the supernatant fluids inoculated intracerebrally in normal monkeys. The acid preparation gave rise to no symptoms, although the alkaline produced characteristic poliomyelitis. These results are presented in Table II.

IMMUNIZATION WITH MIXTURES OF POLIOMYELITIS VIRUS AND ALUMINUM HYDROXIDE

By C. P. RHOADS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, December 1, 1930)

The inactivation of filtrable viruses by adsorption on particulate or colloidal suspensions has been the subject of a number of communications. This paper presents observations on the inactivation of poliomyelitis virus by aluminum hydroxide and on the effect of virus so treated, when used for the production of active immunity.

Levaditi and Nicolau (1), in 1923, reported that they were able to render vaccine virus non-infectious by treatment with precipitated bismuth and thorium. Lewis and Andervont (2) made an extensive study of this phenomenon in 1927. They employed the viruses of vaccinia, fowl-pox, and Rous sarcoma, adsorbed on kaolin, kieselguhr, charcoal, carmine, and aluminum hydroxide. These substances were investigated for both adsorbing and inactivating power and all were found to be effective, although the degree of activity varied somewhat. No attempts to induce immunity by the use of such non-infectious material were reported.

The experiments recorded in this communication were part of a series carried out in a study of various methods for the production of an immunity to experimental poliomyelitis in monkeys.

Method

The aluminum hydroxide preparation employed was that described by Willstätter (3) as Type C to contain 22.5 gm. of aluminum per liter. Since this suspension is relatively unstable, only freshly prepared material was used. The virus was a Berkefeld N filtrate of fresh monkey poliomyelitic spinal cord, made to a 5 per cent suspension in physiological saline, the strain being the "pooled mixed virus" described in previous communications (4). Mixtures containing various proportions of virus and suspension were prepared and shaken in a mechanical device for 30 minutes. They were next allowed to stand 30 minutes at room temperature, before inoculation into *Macacus rhesus* monkeys. A simple test showed that the electrolyte content of the saline solution was not sufficient to cause flocculation of the adsorbing agent.

TABLE IV
Immunity Tests of Animals Treated with *Poliomyelitis Virus Inactivated by Aluminum Hydroxide*

Monkey No.	First test for immunity						Second test for immunity			
	Rest period	Test			Result	Interval	Test			Result
	days	Virus	Route	Total doses	In-terval		Virus	Route	Amount	
8	30	Died of intercurrent disease			day					
9	30	Mixed virus glycerolated 10% suspension	Nasal	6	3	1	Mixed virus	Intra-cerebral	0.01 cc. filtrate	No symptoms
10	30	Mixed virus glycerolated 10% suspension	Nasal	6	3	1	Mixed virus	Intra-cerebral	0.01 cc. filtrate	No symptoms
11	30	Mixed virus glycerolated 10% suspension	Nasal	6	3	1	Mixed virus	Intra-cerebral	0.01 cc. filtrate	Typical poliomyelitis
Control		Mixed virus glycerolated 10% suspension	Nasal	6	3	1	Mixed virus	Intra-cerebral	0.01 cc. filtrate	Typical poliomyelitis, 7 days

In the third kind of experiment the virus used was monkey poliomyelitic nervous tissue which had been preserved in 50 per cent

TABLE II
Influence of pH on the Inactivation of Poliomyelitis Virus by Aluminum Hydroxide

No.	Date	Material tested for neutralization	Amount	Virus filtrate	Result
6	11/29	Aluminum hydroxide. Phosphate buffer pH 8.8. Mixture centrifuged. Supernatant fluid inoculated	cc. 0.2	cc. 0.2	Typical poliomyelitis, 7 days
7	11/29	Aluminum hydroxide. Phosphate buffer pH 5.5. Mixture centrifuged. Supernatant fluid inoculated	0.2	0.2	No symptoms

TABLE III
Production of Immunity by Poliomyelitis Virus Inactivated by Aluminum Hydroxide

Monkey No.	Material used in each treatment			Immunizing treatment					
	AlO ₃	Mixed virus glycerolated 5% suspension	Treatment	Route	Total amount	Single dose	No. of doses	Interval	Rest period
8	cc. 1.5	cc. 15	Shaken 30 min. 1 hr. at 20°	Subcutaneous	cc. 75	cc. 15	5	days 3	mo. 1
9	1.5	15	Shaken 30 min. 1 hr. at 20°	Subcutaneous	75	15	5	3	1
10	1.5	15	Shaken 30 min. 1 hr. at 20°	Subcutaneous	75	15	5	3	1
11	1.5	15	Shaken 30 min. 1 hr. at 20°	Subcutaneous	75	15	5	3	1

glycerol for from 1 to 2 months. The infectivity of such material has been found to be lower than that of fresh virus. Tests showed that

jections of poliomyelitis virus so inactivated evince immunity as shown by resistance to intranasal instillation and intracerebral inoculation as well as by the neutralizing power of their sera. The treatments which give rise to the immunity produce no symptoms of disease.

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a mixture of ten parts of a 5 per cent physiological saline suspension of glycerolated virus with one part of aluminum hydroxide was without effect when inoculated intracerebrally in normal monkeys.

For the immunization test a series of four *Macacus rhesus* was treated by 5 sets of subcutaneous injections of such a 10:1 mixture. Each set was composed of 15 cc. of virus suspension and 1.5 cc. of aluminum hydroxide, distributed in multiple blebs. The treatments were given at 3 day intervals; thus each animal received a total of 75 cc. in a period of 2 weeks. One monkey died of intercurrent disease, but no symptoms referable to the inoculations were observed. A rest period of 1 month was allowed to elapse before the animals were bled and the serum stored (Table III).

The first test of immunity consisted of the instillation of 1 cc. of glycerolated virus emulsion in each nostril, a procedure repeated daily for 3 days. All three treated monkeys remained well although a control animal developed typical poliomyelitis.

A second test of immunity was carried out 28 days after the first. In this test direct intracerebral inoculation of fresh virus filtrate was substituted for intranasal instillation of glycerolated material. As a result of this inoculation one of the treated monkeys which had survived the first test developed characteristic poliomyelitis as did the untreated control. The other two animals remained well. These experiments are epitomized in Table IV.

As further indication of the immunity resulting from the inoculation of inactivated virus, the neutralizing power of the treated animal's sera was investigated. A volume of 0.99 cc. of each serum was mixed with 0.01 cc. of fresh virus filtrate, kept 1 hour in the incubator, and overnight in the icebox, and inoculated intracerebrally in a normal monkey. None of these animals showed symptoms, although the control, receiving the same amount of virus alone, developed the characteristic disease.

SUMMARY

Preliminary experiments are presented which indicate that poliomyelitis virus can be inactivated by a certain preparation of aluminum hydroxide. This effect is seen at neutrality and at a pH of 5.5, but not at a pH of 8.8. Monkeys treated by repeated subcutaneous in-

by means of an electric fan to prevent heat from the X-ray tube reaching the animal. The dosage used throughout the experiments was supplied by an outfit known to produce X-rays remarkably constant both in quality and intensity. The tube was operated at 30 kv. and 22 ma. with a target distance of 27.5 cm. The duration of exposure was 10 minutes, giving a dosage which previous experiments had proved to be suberythematous. The technique for holding the animals in place and at a definite target distance has already been described (3).

Heat treatments were given by means of a flat, hollow brass button, $\frac{3}{4}$ inch in diameter and $\frac{3}{8}$ inch thick, through which water from a constant temperature bath was passed. The circulation through the button, from and to the bath, was maintained by means of an electric pump driven at a definite speed. The heat treatment was confined to the portion of skin covered by the button, which was attached firmly to the abdomen of the animal by means of a broad elastic band that encircled the body. Throughout the experiments the temperature of the water in the button was maintained at 46°C.

Experiment 1.—The abdomens of four guinea pigs were shaved and an area exposed for 10 minutes to X-rays of the intensity noted above. The brass button was placed over different parts of the same area, at the following times: immediately, 3 hours, 1 day and 7 days after the X-ray treatment, and water at 46°C. was circulated through it for 10 minutes. The same animals were given the heat treatment at the same time intervals on areas which had had no X-ray exposure, as controls for the heat alone. Consequently, the control exposures to X-rays and heat alone, and the combined exposures to the two agents, were made on each animal.

The areas exposed to X-ray alone developed slight scaling of the skin on or about the 11th day after treatment, and this persisted for 4 days. In the areas exposed to heat immediately after X-ray, definite burns appeared on the 2nd day, and the healing of these burns took from 14 to 24 days. Three of the animals developed burns in the areas where heat was applied 3 hours after X-ray, which did not heal until between the 11th and 14th day. The other animals developed heavy scaling in this area which persisted for 9 days. In the areas exposed to heat 1 day after X-ray, scaling of the skin developed 2 days after heat treatment and lasted for 9 days. Three of the animals showed scaling in the areas exposed to heat 7 days after X-ray, the scaling appearing 3 days after heat treatment and lasting for 2 days. None of the animals showed visible skin changes in the areas exposed to heat alone. The results are graphically shown in Text-fig. 1.

Experiment 2.—The abdomens of four guinea pigs were shaved. The brass button was placed over different parts of this area at time intervals so spaced that 2 spots

THE SKIN REACTIONS PRODUCED BY ALTERNATIONS OF HEAT AND X-RAY AT VARIOUS TIME INTERVALS*†

By JAMES A. HAWKINS, Ph.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, November 3, 1930)

We have previously reported some observations on the effect of exposures to heat and X-rays on the skin reactions of guinea pigs. In the first series of experiments (1), in which the two agents were applied simultaneously, well-marked burns resulted, which healed very slowly, although the dosage of X-ray was not sufficient to cause even a mild erythema, and the heat exposures alone gave only a slight burn in 50 per cent of the animals. In the second series of experiments (2), in which the two agents were applied alternately, in order to determine whether or not one of the physical agents is capable of sensitizing the tissues to the action of the other, well-marked and persistent burns resulted in whichever sequence the agents were applied. It is thus apparent that either type of radiation is capable of augmenting the action of the other.

The present investigation was carried out to determine the duration of the sensitization of tissues produced by one agent to the action of the other.

Method

The character and measurements of the applications of X-ray and heat have been described previously (1-4).

During exposure the animal and X-ray tube occupied separate compartments of a lead-lined cabinet with an aperture for the passage of the rays, closed with thin bristol board. The chambers and partition were kept cool and ventilated

* This investigation was carried out by means of funds from the Rutherford Donation.

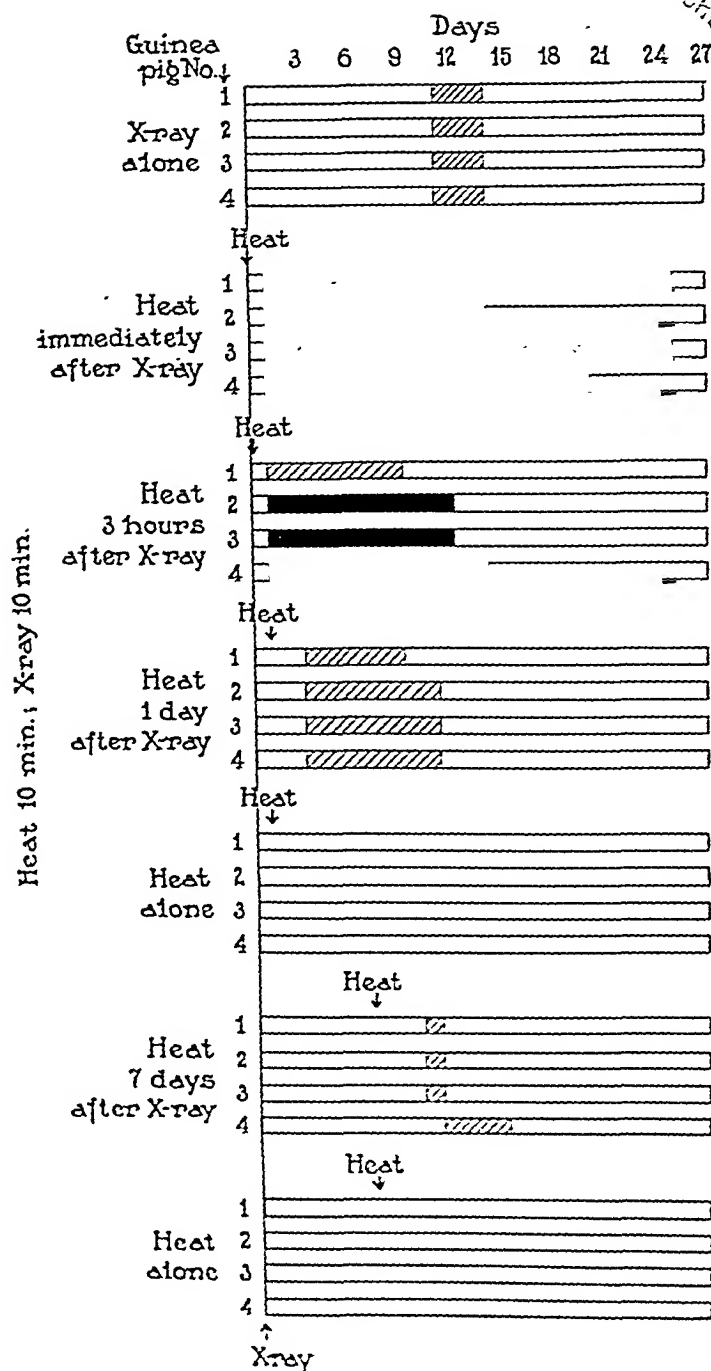
† The experiments reported in this communication were carried out with the cooperation of Dr. Harry Clark.

each were exposed to heat 7 days, 1 day, 3 hours and immediately before the X-ray treatment. Water at 46°C. was circulated through the button for 10 minutes. A whole area containing a heated spot for each period was then exposed for 10 minutes to X-ray of the intensity noted above. Thus the control exposures to X-rays and heat alone and the combined exposures to the two agents were made on each animal.

The areas exposed to heat alone, as controls for the period immediately before X-ray, showed no skin reaction. The areas exposed to heat alone, as controls for the period 1 day before X-ray, developed slight scaling of the skin in two animals on the 1st day, and in the other two on the 2nd and 3rd days. This scaling persisted for 3 days. In the areas exposed to heat immediately and 3 hours before X-ray definite burns appeared on the 2nd day. The healing of these burns took from 10 to 17 days. In the areas exposed to heat 1 day before X-ray slight scaling of skin developed on the 2nd day and persisted for 8 days. In the areas exposed to heat 7 days before X-ray slight scaling of skin appeared and lasted for 5 days. This scaling had disappeared before X-ray treatment was given and no further skin changes resulted. No visible skin changes appeared in the areas exposed to X-ray alone. The results are tabulated in Text-fig. 2.

From these experiments it is seen that there is a very marked difference in the reaction of the areas which received X-ray or heat alone and those exposed to both types of radiation in sequence, a fact that has been shown before (2). The maximum effect of each agent alone was a slight scaling of the skin. There is a marked difference in the areas exposed to both agents where the treatment with one agent preceded the other by 3 hours, as compared with the areas exposed to heat or X-ray alone. A burn invariably resulted in the areas exposed to both agents, when the treatment with one followed the other immediately or in 3 hours. There was only a slight difference in the areas exposed to both agents, where the one preceded the other by 1 day, as compared with the areas that were exposed to heat or X-ray alone. Scaling of the skin developed in these areas and persisted for several days longer than in the areas exposed to heat or X-ray alone. There is no visible augmentation of effect on the skin when the treatment with either agent preceded the other by 7 days.

The sequence in which the agents were applied seemed to make no



TEXT-FIG. 1. Each line represents the changes in an area of skin exposed to heat or X-ray or both. The duration of a burn is indicated by heavy lines, smiling by cross-hatch, while the unshaded lines indicate that there are no visible reactions.

difference in the intensity of the reaction. It is apparent from these observations that either type of radiation is capable of sensitizing the skin to the action of the other, within certain limits.

DISCUSSION

In previous experiments (1) in which the skin of guinea pigs was exposed to X-ray and heat radiation simultaneously, burns were produced which had the appearance of heat burns, but were much more extensive. In a second series of experiments (2), in which the exposures were in sequence, extensive burns resulted, the reaction being the same regardless of which agent was applied first. The fact that burns developed so soon after the treatment, and their general appearance during the first few days, gave them a close resemblance to a heat reaction, but the later appearance, the slowness of healing and the type of resulting scar were more characteristic of an X-ray effect. Thus the lesions produced had some of the features characteristic of the action of both agents.

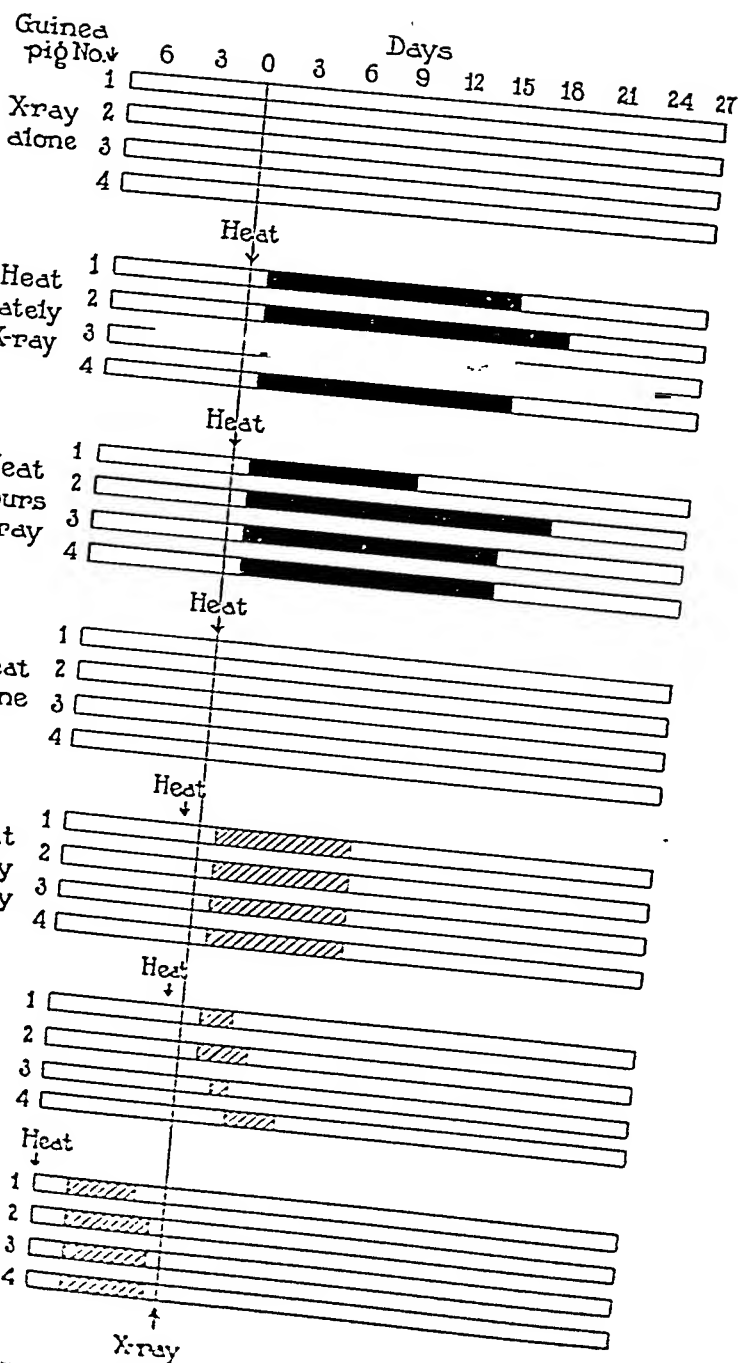
The present experiments were undertaken to see if some insight into the nature of the effect of radiant energy on the living cell could be obtained. Burns similar to those in previous experiments were obtained even when one agent was applied 3 hours after the other. Scaling of the skin resulted when the interval was as great as a day and this scaling was heavier and lasted for a longer time than that produced by either heat or X-ray alone. It can be concluded that the sensitizing effect persisted in some degree for 24 hours. Heat applied 1 week before X-ray caused no visible augmentation of the skin reaction to the latter. This was to be expected. No apparent skin changes followed the application of heat to areas X-rayed 1 week previously, though the effects of the raying were beginning to appear.

It is evident that either type of radiation is capable of sensitizing to the action of the other. The result was the same regardless of whether the heat or the X-ray treatment was given first. No interpretation of the phenomenon is ventured.

SUMMARY

Areas on the abdomen of the same guinea pig were exposed to sub-erythreal doses of soft X-rays, to heat of an intensity below the critical

X-ray 10 min.; Heat 10 min.



TEXT-FIG. 2. For explanation see Text-fig. 1.

dose for the production of burns, and to both radiations in sequence with various time intervals between the exposures.

The only effect of exposure to X-ray or heat alone was a slight scaling of the skin. The areas exposed to heat and X-radiation developed well-marked and persistent burns when the exposure to one agent was made within 3 hours of the other. Scaling of the skin developed when the exposure to one agent was made 1 day after the other. This scaling was more marked and lasted longer than the scaling produced by either agent alone. The results were the same no matter in which sequence the agents were applied.

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part responsible for the marrow hyperplasia although the phagocytosis of silver by marrow cells may also be concerned.

It is possible, although very difficult, to produce a condition of aplastic anemia in dogs by means of Roentgen radiation. At the same time however there is almost complete disappearance of white cells and platelets from the blood. Multiple hemorrhages then develop due to platelet deficiency or to endothelial injury or both. Terminal infections (agranulocytosis) are all too frequent. It is obvious that this type of experimental aplastic anemia with the hemorrhagic complications is not suitable for the proposed study of the circulating red cells.

Method

Our studies were made on healthy and well nourished full-grown dogs. All of them were under observation at least 2 weeks before the experiments were begun. A diet of mixed kitchen scraps was used generally, but a few of the animals were fed small amounts of raw beef after they had become inactive and refused to eat the usual food.

All the blood studies were made on blood obtained by hypodermic puncture of the jugular vein. The red blood cell hematocrit was determined before the silver injections were begun and at 1 or 2 day intervals thereafter. The same hematocrit tube was used for each dog throughout. Approximately 10 cc. of blood were added to 2 cc. of 1.6 per cent sodium oxalate solution, and centrifugalized at 2,400 revolutions per minute for $\frac{1}{2}$ hour.

In these experiments we employed only colloidal silver. The preparation used was collargolum, a crystalline material supplied by the Heyden Chemical Company. It is said to contain 85.87 per cent of metallic silver and a small percentage of albumen. A 1 per cent emulsion of this was prepared with distilled water, filtered, and kept in a brown bottle in a refrigerator. As an additional precaution against its disintegration, the stock bottle of the crystals was also kept in a refrigerator. Just prior to each injection the emulsion was carefully warmed in the syringe to approximately body temperature. It was slowly introduced into a jugular vein. The dosage varied, as described in the following protocols.

EXPERIMENTAL OBSERVATIONS

A single intravenous injection of 500 mg. of collargol is followed as a rule by death in less than 12 hours without convulsions or clinical evidences of shock. This single large dose produces moderate congestion and marked edema of the lungs.

I. EFFECTS OF THE INTRAVENOUS INJECTION OF COLLOIDAL SILVER UPON THE HEMATOPOIETIC SYSTEM IN DOGS

By SAMUEL S. SHOUSE, M.D., AND GEORGE H. WHIPPLE, M.D.

(From the Department of Pathology of the University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

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In this laboratory during several years much experimental work has been done to study the effect of diet upon blood regeneration in dogs with anemia caused by bleeding (6). The anemia is continued at a uniform level of about one-third normal by repeated blood withdrawal from the jugular vein. Under such conditions the bone marrow of all bones shows *extreme hyperplasia* and presumably is functioning at maximal capacity due to a continued maximal demand associated with severe anemia.

If an *aplastic anemia* with great depletion of bone marrow cells could be produced experimentally, a rare opportunity would be offered to study the life cycle of the red cell under carefully controlled conditions. This ideal condition has never been produced experimentally in the dog although Muller (3) reports suggestive observations in the rabbit. We had hopes that by means of colloidal silver or Roentgen radiation or both it might be possible to cause a type of aplastic anemia which would yield much valuable information.

As sometimes happens in such experiments we were able to take a step toward our goal of experimental aplastic anemia but so many complicating factors developed simultaneously that we were unable to get any clear-cut evidence as to the life cycle of the red blood cell in the circulation of the dog.

As is evident from the protocols given below the *colloidal silver* injections in dogs cause no aplasia of the marrow but rather a distinct *marrow hyperplasia*. Moreover there is some hemolysis which in some way must be related to the silver injections. This hemolysis may be in

The following experiment shows the fluctuation of the hematocrit and white blood cells under *prolonged administration* of silver in moderate amount.

Dog 27-90.—A female, weighing 14 kg. During the first 28 days she received small doses of 20 to 50 mg. of collargol at intervals, making a total of 150 mg. During that time there was moderate leucocytosis and an increase in the red cell hematocrit from 46 per cent to 52 per cent. Between the 28th and 36th days she was given 900 mg. collargol in doses of 100 to 300 mg. each. The silver injections were stopped then because of anorexia and loss of weight. By the 49th day she had lost 4 kg. in weight, the hematocrit was down to 39 per cent, and a nasal discharge was observed. Her general condition and appetite improved after cessation of the silver injections. On the 62nd day the red cell hematocrit was 36 per cent and the leucocyte count was 13,600. 400 mg. of collargol were given that day. On the 70th day the hematocrit was 29 per cent and the leucocyte count was 13,400; nevertheless, she was given another 400 mg. dose of collargol. 5 days later the hematocrit was 24 per cent, the leucocyte count 9,500; the white count continued between 9,000 and 15,000 and she remained very lethargic until the end. The hematocrit was 18 per cent from the 101st until the 115th day. On that day she was given 300 mg. collargol, although her condition was bad at the time. 3 days later the hematocrit had risen to 27 per cent and it was 22 per cent on the 122nd day. At that time she was given 600 mg. of collargol, making a total of 2,600 mg. in 4 months. She was found dead the next morning. The day before death she weighed 9.8 kg., whereas the initial weight had been 14 kg. Hemolysis was present during and for several days after each course of collargol.

Necropsy showed an extreme emaciation; slimy exudate in the nose. The spleen had a dark slate color, and microscopic examination showed very numerous clumps of yellowish brown granular particles (silver), chiefly intracellular. The latter were not colored blue when stained for iron. A few megakaryocytes were seen. The liver was chocolate brown, and showed numerous yellowish brown granular particles in the sinusoids, most of which were in large phagocytes. There were extensive central necroses in which there were moderate numbers of mononuclears and very prominent deposits of the granular material. The pancreas had a gray color but microscopic examination showed many yellowish brown granular particles (silver) scattered in the stroma. The kidney sections revealed a small amount of similar particles in the glomerular tufts, which did not turn blue with the iron stain. A lymph node showed numerous clumps of these particles. The femur marrow was very hyperplastic, and all the fat had disappeared; there were abundant parent marrow cells and several mitotic figures were noted. The vertebral marrow was similar to that of the femur, but in addition it contained a moderate amount of the yellowish brown granular particles.

The following protocol shows the results of 3 courses of silver, followed each time by an hemolysis, an anemia, and later by recovery.

Doses of 1,300 to 1,500 mg. of collargol are tolerated if divided and given at the proper intervals over a period of 3 to 7 days. There is no conspicuous tolerance afforded by repeated doses nor is the effect cumulative in the strict sense. Single doses of 200 to 300 mg. are well tolerated. Death usually follows a single large dose even after several preliminary smaller doses. Evidence of hemolysis is always present. The following brief protocol gives the outline of a typical short experiment:

Dog 27-249.—A male Dalmatian coach, weighing 23 kg., was given 400 mg. of collargol intravenously in divided doses, on the 1st day of the experiment. The next day he received two doses of 200 mg. and 300 mg. respectively. The 3rd day he was given one dose of 400 mg. making in all 1,300 mg. No reaction occurred during the next 40 minutes after the injection, but 4 hours after the last injection he was found dead. Necropsy revealed marked edema and moderate congestion of both lungs. The spleen and liver had a dark slaty color, and their reticulo-endothelial cells contained coarsely granular golden brown pigment (silver). The bone marrow showed slight hyperplasia and many large mononuclear cells filled with brown pigment.

In these short experiments the hematocrit drops 10 to 14 per cent and on examination the bone marrow may present a normal appearance or a hyperplasia. This is illustrated by the following protocol in which a large total dose was given over a somewhat longer period.

Dog 28-114.—A male, weighing 19.4 kg., received rather small divided doses of collargol during the first 6 days, but the next 3 days he was given single daily doses of 300 mg., making a total of 1,900 mg. He was inactive and had not eaten when the last dose was given; his condition was unchanged 8 hours later but the following morning he was found dead. Necropsy revealed considerable edema and congestion of the lungs. The spleen and liver had a dark brown color; sections showed abundant coarsely granular golden brown material (silver) in the reticulo-endothelial cells, and fine brown particles in the parenchyma cells of the liver. The femur marrow showed hyperplasia and contained numerous deposits of coarsely granular golden brown pigment. The initial hematocrit of 54 per cent had dropped to 47 per cent on the 8th day.

Two dogs receiving smaller individual and smaller total injections over 2 to 3 weeks developed distemper and death was due to this intercurrent infection. The hematocrits dropped 20 per cent and a marked loss of weight occurred. The bone marrow was either normal or slightly hyperplastic.

pulmonary edema. Chemical analysis of the tissues of their patient showed no silver in the lungs, a definite trace in the kidneys, and an abundance in the spleen and liver. Those results are in harmony with the microscopic findings in our animals, in which the endothelial cells of the liver and spleen contained large deposits of the silver. The spleen in our dogs usually presented a dark slaty color. An iron stain was done on several cases, which indicated that these deposits did not contain any hemosiderin. Voigt (5) mentions a rabbit weighing 2 kg. which was given an intravenous injection of 120 mg. of collargol. There was no immediate reaction, but the next morning it was found dead. Autopsy showed definite edema of the lungs; the liver and spleen had a black color, and contained abundant accumulations of dark particles; the kidney contained none but the reticulo-endothelial cells of the bone marrow were laden with silver particles.

Herzog and Roscher (1) injected collargol into rabbits and obtained a rapidly developing anemia. More recently Muller (3) has found that colloidal silver, administered intravenously to rabbits, produced first an erythrocytic hyperplasia, followed by a condition which resembled aplastic anemia. Aplasia of the erythroblastic bone marrow seemed to be present before there was an appreciable decrease in the number of circulating red blood cells. She concluded that the anemia was caused by the endothelial cells of the bone marrow forming clasmatoocytes at the expense of the development of the erythrocytes.

It is noted that three of our dogs contracted distemper soon after the silver injections were begun. We feel justified in attributing the onset of this disease to the lowered vitality of the animals which resulted from the silver intoxication. It is possible that these were latent cases of distemper and that an exacerbation occurred when the resistance was lowered. In this connection it may be noted that 30 years ago collargol was used as an internal antiseptic under various conditions.

During the first 5 to 8 days of the injection of collargol the red cell hematocrits drop 7 to 14 per cent, although the bone marrow may appear normal or moderately hyperplastic at autopsy. Although the animals which contracted distemper have shown a decline in the hematocrit of 20 to 23 per cent, the bone marrow seemed normal or slightly hyperplastic. In animals which were studied almost 4 months a marked anemia was observed, with a terminal hematocrit of 22 per cent to 28 per cent, but this was accompanied by a hyperplastic bone marrow. Thus, in none of the animals did we find the slightest sign of bone marrow *aplasia*; indeed quite the opposite picture was presented, for there were numerous parent cells which seemed perfectly capable

After the second course of silver the hematocrit was so low (20 per cent) that a transfusion was given. This apparently had only a temporary effect. The peripheral destruction of the blood and the hyperplasia of the marrow are the outstanding findings.

Dog 28-178.—A male, weighing 15.5 kg., received between 50 mg. and 125 mg. of collargol daily for 13 days, making a total of 1,150 mg. The original hematocrit of 54 per cent had dropped to 25 per cent on the 22nd day, and the weight had fallen to 12.9 kg. on the 25th day. By the 57th day the hematocrit had risen to 32 per cent. He was then given 70 mg. of collargol daily for 10 days. On the 71st day the hematocrit was 20 per cent; that day a transfusion was done which brought the red cell hematocrit up to 30 per cent. On the 86th day it had declined to 21 per cent and another transfusion was done which increased the red cell hematocrit to 26 per cent. On the 97th day the red cell hematocrit was 28 per cent and his general condition seemed very good. Because of a rising hematocrit a third course of silver was given, consisting of 150 mg. for 4 days, on the 98th to 101st days. On the 99th day the hematocrit was 34 per cent and on the 101st it was 33 per cent—that day he was inactive and did not eat. This condition continued, he gradually became weaker, and died on the 105th day. The hematocrit was 28 per cent the day before death.

The plasma showed hemolysis from the 4th to the 15th day, but had regained its normal color by the 17th day. It had a lemon color after the second course of collargol, even before the transfusion. It was very red during the last 3 days of life, following the third course of silver. The leucocyte count dropped to 4,600 on the 23rd and 24th days, but thereafter it remained between 8,000 and 13,000.

Necropsy revealed a dark brown spleen in which microscopic examination showed very numerous clumps of coarsely granular golden brown particles (silver). The liver showed many focal necroses with an infiltration of mononuclears and the Kupffer cells were filled with coarsely granular golden brown particles. Bone marrow from the femur, vertebrae, and ribs showed marked hyperplasia, with no death of the parent cells.

DISCUSSION

Five of the acute experiments were characterized by edema and congestion of the lungs, obviously due to the acute toxic action of the massive doses of silver. There is nothing specific about this reaction which is typical of the heavy metals.

Patein and Roblin (4) reported a human death which occurred within 2 hours after an intravenous injection of 50 mg. of collargol. The autopsy showed acute

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of producing mature blood cells. Likewise the human cases which died after repeated injections of collargol presented moderate anemia and hyperplasia of the bone marrow.

This anemia observed in our experiments is probably in large part due to an increased destruction of the circulating red blood cells, since we observed hemolysis in the plasma of several of the animals. Muller (3) stated that she obtained no evidence of injury to the red blood cells in the peripheral circulation, but she notes a rapid decrease in the number of erythrocytes in one rabbit, in which the spleen was found loaded with pigment. Motohashi (2) found marked phagocytosis of the red blood cells in rabbits following the injection of collargol. Voigt (5) states that after being injected intravenously, the colloidal silver is gradually changed into the ionized form, and he attributes collargol poisoning to this transformation.

CONCLUSIONS

1. Colloidal silver has no specific action on the bone marrow in dogs but is a systemic poison which may cause anorexia, weakness, loss of weight, anemia, and death.
2. Hemolysis can be demonstrated after large doses of colloidal silver and the anemia presumably is due in part at least to a destruction of red blood cells in the peripheral circulation.
3. The colloidal silver, injected intravenously, is deposited as granules almost exclusively in the cells of the reticulo-endothelial system after the manner of particulate substances.
4. Repeated injections of non-lethal amounts of this substance are invariably followed by hyperplasia of the bone marrow. In no case was aplasia found.
5. Large single doses of this material cause rapid death in 12 hours or less characterized by pulmonary edema and congestion.
6. An initial increase in the number of erythrocytes and leucocytes may occur following smaller amounts of silver, but repeated injections cause a considerable anemia, without a definite increase in the leucocytes and with no signs of blood platelet deficiency.

megakaryocytes. Furthermore it indicates a short life cycle for the blood platelet in the circulation.

It may be argued that the radiation injures the capillary endothelium which therefore is responsible for the tissue bleeding. It should be recalled that the tissue bleeding occurs in the gastrointestinal muscle coats which are shielded with lead and in addition are known to be resistant to direct radiation (12).

All in all, the sudden bleeding into the tissues in the last day of life seems to be the most important factor in causing death on the 8th or 9th day following radiation of the skeleton. Dogs will survive this massive radiation if a small area of the skeleton escapes the field of radiation. This again suggests the possibility that platelets may be contributed by these uninjured areas in sufficient numbers to prevent the extensive purpura. However it seems wise to admit that other factors may be concerned—some unknown.

Finally we wish to emphasize one conspicuous feature. Death occurs on the 8th or 9th day with great regularity when the standard filtered radiation is given over all bones. It seems as difficult to explain this *latent period* satisfactorily as it is to give a comprehensive explanation for the *delay* in breakdown of the skin ("x-ray" burn) 2 to 3 weeks after radiation. The clinical picture is that of a normal healthy dog—active, good appetite, no loss of weight for 6 or 7 days after radiation. Then suddenly there is loss of appetite and perhaps a little vomiting but no clinical evidence of severe intoxication until a few hours before death.

It should be pointed out that the average of many experiments shows a drop in red cell hematocrit from a normal of about 50 per cent to a level of about 40 per cent within 7 days. One might argue that the exposure to Roentgen radiation caused complete cessation of red cell production and that this loss of circulating red cells represented the normal wear and tear of circulatory function. If this were so then the life cycle of the red cells under such conditions would approximate 5 weeks. There are too many confusing factors to justify a claim that such experiments are conclusive, much as we would like to establish the life cycle period of the normal red cell in the circulation of the dog.

Space will not permit a careful review of the experimental and clinical observations which relate indirectly to the experiments given

II. APLASIA OF MARROW AND FATAL INTOXICATION IN DOGS PRODUCED BY ROENTGEN RADIATION OF ALL BONES

By SAMUEL S. SHOUSE, M.D., STAFFORD L. WARREN, M.D., AND
GEORGE H. WHIPPLE, M.D.

(From the Departments of Pathology and Radiology of the University of Rochester
School of Medicine and Dentistry, Rochester, N. Y.)

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Our main objective in these experiments was the production of *aplasia of the bone marrow*. In this we were successful but at the same time there developed a peculiar and fatal intoxication which terminated the experiments. This peculiar intoxication associated with exposure of the bones to Roentgen radiation has not been previously described and holds some interest for workers in this field.

Many factors may be concerned in the cause of death in these animals. One may believe that the destruction of this mass of cells in the marrow may free split products which are poisonous and cause the fatal intoxication. It is fair to say however that the cell breakdown comes early whereas the serious intoxication comes only the day before death when practically all marrow cells and circulating leucocytes have vanished. The rise in non-protein nitrogen in the blood is also limited to the last day or two of life.

The extraordinary drop in circulating *white blood cells* (agranulocytosis) may be concerned with the fatal intoxication in some experiments where we find massive colonies of bacteria growing in the lung tissue. There is total lack of tissue and white cell reaction to such bacterial invasion.

We recall the drop in *blood platelets* which practically disappear from the circulating blood the day before death. This may be largely responsible for the bleeding into the tissues which takes place during the last 24 hours of life. There is evidence that practically all megakaryocytes of the marrow are destroyed by the radiation and this gives confirmatory evidence to indicate the origin of the platelets from the

was obtained in pure form in ampules. This drug can be given intravenously and is followed promptly by anesthesia with satisfactory experimental results.

The following procedure was finally worked out and used in the complete experiments reported below. $\frac{1}{4}$ grain of morphine was given subcutaneously, $\frac{1}{2}$ hour before the sodium amytal. Sodium amytal, estimated on the basis of 35 to 40 mg. per kilo of body weight, was then injected at the rate of 10 mg. per minute, until the animal was sound asleep. Enough was injected to reduce the eyelid reflex but not to abolish it. Complete anesthesia lasted usually from 5 to 6 hours. The animal was kept in a warm room, wrapped in cloths, and was fully awake by the next day.

Usually two dogs of equal size and weight were exposed to the radiation at one time, since our equipment consists of a deep therapy couch with two portals equally distant from the target. The maximum portal size is 30 cm. square, so that the exposure had to be divided into two portions in order to cover the whole body of the animal. Both exposures were given at one sitting.

When completely under the anesthetic, the animals were carefully adjusted so that the upper half of the whole body including the head and the paws and forelegs and the trunk down to the costal margin was covered by the boundaries of one portal. After the dose was given over this area, the animal was shifted and placed on its side over the portal. A piece of lead $\frac{1}{8}$ inch thick was carefully adjusted to protect completely all of the abdominal viscera in such a manner as not to shield the lumbar spine or the pelvis. This was controlled by fluoroscopic observation. The hind legs and paws, the tail, pelvis, and lumbar spine up to and slightly over the edge of the portion radiated at the previous exposure were carefully included within the field of radiation.

The factors of radiation were: target skin distance, 60 cm.; filter, $\frac{1}{2}$ mm. of copper, 1 mm. of aluminum; 200 kilovolts; 35 milliamperes, watercooled Coolidge tube. The time of exposure varied from 20 to 80 minutes though the usual standard dose was given in 40 minutes, equivalent to 1,050 milliampere minutes. This dosage, which is three times the human epilation dose, always gives an epilation in a dog in 3 weeks with a very mild erythema which is only visible in white or through unpigmented areas of the skin. With repeated trauma such as might occur in the axilla, there is occasionally a slight amount of desquamation of the superficial layers of the skin. The greatest variation in the voltage, as indicated by the sphere gap, in any of the experiments was from 190 to 210 kilovolts. Usually, however, the kilovoltage was maintained throughout the experiment at a constant level as indicated by ionization chamber, electrostatic volt meter, and the sphere gap readings.

On the 2nd day after the radiation, hematocrit and blood counts were again done and continued every other day until death occurred. When the animals became moribund, they were etherized and a complete autopsy done. Sections of all the organs, the bones, and the bone marrow were saved for microscopic study. These were fixed in Zenker's solution and cut and stained with hematoxylin-eosin in the usual manner.

below. Those specially interested are referred to papers by Lacassagne (5), Minot and Spurling (7), Krömeke (4), Warren (11), Linser and Helber (2, 6), Heineke (1), Krause and Ziegler (3), Piney (8, 9). The smaller laboratory animals (especially the rabbit) are used by most workers and the dog but rarely.

The acute changes due to radiation in massive doses as recorded in the literature are few in number. The leucopenia with general reduction of the circulating leucocytes and the disappearance of the lymphocytes is well established. There is some disagreement about the immediate leucocytosis occurring a few hours after radiation but the later workers seem to have demonstrated it in rabbits. The leucopenia commences 24 hours or so after the radiation. The single report (Lacassagne) of purpura in new-born rabbits just before death does not seem to have been observed by others. Most reports do not show any disturbance of the platelets or adult red blood cells. Recently Wright and Bulman (1929) (13) have described changes in the platelets in prolonged experiments on the cat. Injury to the lymph nodes and spleen is well known. Many authors state that the marrow injury is extreme yet picture or describe many marrow cells remaining in sections after the radiation. Thorum X given by injection may bring about changes very like those described in our experiments on dogs with massive doses of radiation. We refer to the papers of Valeeff (1927) (10).

Method

In these experiments, normal, strong, healthy stock dogs were used throughout. Care was taken to select animals immune to distemper since the prolonged drop in the temperature following the amytal anesthesia often precipitates an attack of distemper or some acute respiratory infection which usually terminates the experiment. The animals were kept on a general diet and were given as much food and water as they would take.

Preliminary white and red blood cell counts, cover slip blood smear preparations, and hematocrits were made in every case during the 2 or more days of observation before the radiation was administered.

Radiation was at all times given while the animal was under amytal anesthesia. Complete anesthesia was always necessary in order to keep the animal properly located over the portal and properly shielded. At first the amytal was administered intraperitoneally, but often the induction of the anesthesia was prolonged and uncertain. Through the courtesy of Eli Lilly and Company, sodium amytal

of the peculiar intoxication and marrow aplasia caused by this standard radiation.

This experiment shows the typical course of events with death 9 days after radiation of the whole skeleton. Radiation 1,050 milliamperere minutes given over upper half and again over the lower half of the skeleton with lead shielding of the abdominal cavity. Leucopenia, platelet deficiency, and terminal purpura are conspicuous features.

Dog 28-257.—A young brown female mongrel, well developed, well nourished, and active. Weight 14.1 kg.

6.19.29. Hematocrit 46 per cent; plasma pale lemon colored.

6.20.29. Active and lively. Appetite excellent.

6.21.29. Active and lively. Appetite excellent. Hematocrit 47 per cent, plasma clear straw colored. W. B. C. 15,000; 15,850. Smear of the blood shows the red cells to be of good color. There is some variation in size but no other abnormality. Platelets are numerous. The differential is essentially normal: polymorphonuclears 85 per cent, eosinophiles 6 per cent, large mononuclears and transitionals 6 per cent, small mononuclears 3 per cent. No abnormal cells are noted. 8 p.m. *Deep therapy* under amytal anesthesia, 35 mg. per kg., administered intraperitoneally. 1,050 M.A.C. laterally over the upper half of the right side of the body including the head and extremities to the costal margin. 1,050 M.A.C. laterally over the lower half of the body from the costal margin to and including the tail and extremities. The abdomen is protected by a sheet of $\frac{1}{8}$ inch lead.

6.22.29. Alert but still ataxic from the anesthetic.

6.23.29. Very active and playful. Appetite excellent.

6.24.29. Active—condition and appetite excellent. Hematocrit 41 per cent, plasma somewhat red. W.B.C. 4,850; 4,900.

6.25.29. Active, appetite and condition excellent.

6.26.29. Clinically normal.

6.27.29. Active and alert. Anorexia. Hematocrit 36 per cent, plasma clear straw colored. W.B.C. 0; 25 (checked).

6.28.29. Seems alert and active but nothing eaten.

6.29.29. Condition seems good. Anorexia. Small amount of vomitus in cage (undigested food). No evidence of hemorrhage. Hematocrit 37 per cent, plasma clear straw colored. W.B.C. 25; 50. The layer of W.B.C. in the hematocrit tubes on 6.27 and 6.29 was so thin as to be hardly more than a visible trace. On 6.29 smear only five polymorphonuclear neutrophiles and one disintegrated white blood cell were found on careful search of the two parts of a cover slip preparation. Platelets were nowhere visible. The red blood corpuscles showed pallor but little other abnormality.

6.30.29. 9:45 a.m. Found dead, body warm, slight rigor mortis.

In order to control the peripheral destruction of adult red blood cells by the radiation given, a fragility study was done as follows. Blood was withdrawn from a normal dog and placed in small glass bottles containing a small amount of dry citrate to prevent coagulation. One bottle was kept protected from radiation behind a lead screen in the same room so as to control both the temperature and radiation changes. One bottle was radiated 1 hour, another $1\frac{1}{2}$ hours, and another 2 hours, making a total of 2,100 milliamperere minutes, 3,150 milliamperere minutes, and 4,200 milliamperere minutes respectively; no filter except the glass, 57 cm. target-bottle distance, 200 kilovolts, 35 milliamperes. The filtration of the glass is negligible since these bottles are barely visible upon a fluoroscopic screen and are not as dense as the blood which they contain. These doses are far beyond those given to the dogs treated over the bone marrow and the absence of filters brings into play all of the wave lengths in the unfiltered beam of 200 kilovolts. There was no hemolysis of the cells in any of the bottles. The routine fragility set-up was used and no definite change in the response of the cells to either hypotonic or hypertonic solution was found.

EXPERIMENTAL OBSERVATIONS

A sufficient number of dogs was used in this study but some experiments were incomplete in one way or another. At times the shielding of the intestinal coils was inadequate and intoxication with diarrhea developed as a result of injury of the intestinal mucosa by radiation. This has been described elsewhere (12) and such experiments were excluded from this series. Other animals received sublethal doses and ultimately recovered. Some animals showed recovery due to the fact that some areas of the skeleton escaped the radiation. These experiments occurred in the early stages of the work when the anesthesia was not deep enough to insure complete immobility. Many animals developed acute respiratory infections or distemper which terminated the experiment before the typical intoxication due to the radiation developed. The leucopenia was probably largely responsible for these infections.

The amount of heavily filtered radiation which was uniformly successful in producing the acute, fatal intoxication was 1,050 milliamperere minutes. Less than this or 700 milliamperere minutes was occasionally followed by a fatal result. More than 1,050 milliamperere minutes caused no appreciable change in the course of events.

We record three typical experiments which are fair examples of a considerable group. We believe these experiments give a fair picture

morphonuclear cells or phagocytes here or elsewhere in the lung sections. In the portions of the lung free of hemorrhage the capillaries are engorged but the alveoli are intact, and the bronchi and larger vessels seem normal.

The *liver* shows a rather marked congestion of the central veins. No hemorrhages noted.

The *kidneys* show an occasional small hemorrhage into the tubules, otherwise they are normal.

In the *adrenal* in some areas there is a rather diffuse invasion of all of the spaces between the cell columns by red cells. Many small areas show focal autolysis of moderate degree.

The *stomach* aside from a massive hemorrhage in the submucosa is not abnormal.

In the *jejunum*, a cross section shows hemorrhage into the villi, into the muscularis mucosae, and occasionally into the muscle coats. The mucosa is seen to be lifted from the muscularis mucosae by the hemorrhage in many places. There is edema of the areolar tissue.

In the ileum the cells of the mucosa show occasional pyknosis and frequent mitotic figures, probably the result of moderate injury by the radiation. Extensive submucosal and intra-mucosal hemorrhage is present and in several places the mucosa has disappeared probably due to the loosening of its attachments by hemorrhage. In the section of ileum which was much dilated and thinned out, the mucosa has been stripped off over a large area. A few of the mucosal cells show pyknosis and slight injury from the radiation. There is some hemorrhage into the villi remaining in this section. The lead shielding of the intestine was not complete in this small area.

The *spleen* appears to have lost much of its substance so that the trabeculae seem more prominent and compact than usual. The germinal centers are reduced in size and contain paler cells more widely separated than usual. The pulp cells are pale and show occasional pyknosis and frequently mitoses. An occasional eosinophile is present. Red cells have infiltrated many areas extensively, involving both the pulp and germinal centers indiscriminately. Occasional phagocytes are seen containing what appears to be shrunken red cells or fragments of red cells. Apparently this has occurred quite recently.

A mesenteric lymph node contains many hemorrhagic areas in the edematous areolar tissue and among the pulp cells. There is occasional necrosis of the germinal centers where they can be identified. There are large numbers of very large giant cells and large phagocytes at the sites of the germinal centers which have been infiltrated by red blood cells. The phagocytes are often distended with 1 to 3 full sized red blood cells.

8th rib on the left and right shows hemorrhage into the fat. The connective tissue framework and the blood vessels represent practically all of the visible cells with the exception of an occasional normoblast.

The *cervical, thoracic, and lumbar vertebrae* have a similar paucity of bone marrow elements, the normoblasts being seen occasionally and undifferentiated

Autopsy at once. The body is well developed and nourished. The skin and hair are apparently normal. There is a small superficial ulceration of the left side of the mouth. Both nasal passages and the accessory nasal sinuses are filled with foul semi-solid grayish material mixed with blood clots. There is no ulceration of the mucous membranes.

The pleural and pericardial surfaces both visceral and parietal show numerous small hemorrhages. There is no free blood in either thoracic cavity or in the pericardial sac. The peritoneal surfaces are smooth and shiny.

The heart shows several ecchymoses in the epicardial fat and endocardium. The muscle is in good condition.

Lungs: There are several small solid hemorrhagic areas in each cut section of every lobe. The middle lobe of the right lung is entirely filled by dark red blood. The spleen appears congested but shows no gross hemorrhage.

The oesophageal mucosa shows many tiny ecchymoses. The stomach contains about 200 cc. of a dark brown thin liquid and the mucosa shows a few ecchymoses. The duodenum appears to be normal. The jejunum and ileum show a few ecchymoses scattered along in the mucosa. There is a section of the lower ileum which shows extensive hemorrhage into the muscle and intestinal wall. In the central portion of this segment the intestine is dilated and thin walled, and the mucosa is desquamated from its surface for a length of about 6 to 8 cm. The mucosa contains a small amount of blood-tinged mucus.

The colon shows many ecchymoses and contains a moderate amount of blood-tinged mucus. There is a section about 8 to 10 cm. long in the lower third where the wall of the colon shows extensive hemorrhagic infiltration, causing the wall to be doubled in thickness.

The lymph nodes in the mesentery are enlarged (3 x 2 cm.) and show considerable hemorrhage within their structure.

The bladder shows a few ecchymoses in the mucosa, otherwise it is normal. The kidneys aside from a moderate number of hemorrhages in the cortex are normal in appearance.

The bone marrow of the long bones is fatty and the marrow contains liquid blood in considerable quantity. That of the ribs is quite red and is mixed with much liquid quickly-clotting blood. The vertebrae are grayish red and show some little cellular material but mostly free blood.

The brain, thyroid, pancreas, liver, adrenals, ovaries, uterus, and other structures show no hemorrhages or other abnormalities.

Microscopical sections were made from all tissues. Organs presenting no features of interest are not mentioned.

The *lungs* in some places present a normal aspect, in others a slight amount of atelectasis, but in most sections red cells are numerous in the alveoli. There are frequently solid masses of bacteria scattered in among the larger hemorrhagic areas usually fairly well in the center of the densely packed red cells. There is usually considerable destruction and autolysis of the alveolar cell remnants and the red cells adjacent to these bacteria but there is a notable absence of any g-

about 6 cm. long just above the ileocaecal valve. The mucosa has not been injured by the radiation but the changes are due to hemorrhagic infiltration. The remainder of the gastrointestinal tract and the oesophagus appear entirely normal.

The *bone marrow* of the long bones, ribs, and vertebrae shows abundant fat but aside from a slight pinkish tinge there is no evidence of free blood or hemorrhage.

The heart, lungs, liver, pancreas, spleen, kidneys, adrenals, bladder, uterus, and ovaries are free from gross hemorrhages and apparently normal.

Microscopical sections made from all tissues.

The *lungs* show many alveoli containing free red blood cells. The epithelium of many of the smaller bronchi is also covered with a layer of red blood cells. There is no massive hemorrhage. Some atelectasis is present here and there and in these areas the capillaries are engorged. There is no evidence of a pneumonic process.

The *liver* shows some congestion, with engorgement of the central veins. There is a small amount of focal central necrosis in many of the liver lobules. Around the hyalinized cells the liver cells are pale and show frequent mitoses. Invasion by phagocytes has not occurred and the rest of the liver cells seem normal.

In the *kidneys* the glomeruli seem congested. Many of the tubules contain a large number of red blood cells. Aside from a moderate amount of postmortem autolysis there is no other deviation from the normal.

Sections from the small *intestines* are somewhat autolyzed and show occasional small areas in which the mucosa has been injured by the radiation. There is a considerable amount of hemorrhage into the smooth muscle bundles, into the areolar tissues, the submucosa, and under the peritoneum. This does not seem associated with the mild injury to the mucosa which is strictly localized to a small area. The capillaries are dilated and congested everywhere.

In the *spleen* the trabeculae seem more prominent than usual due to a generalized reduction of the pulp substance. The germinal centers are greatly reduced in size and number of cells. The sinuses are filled with blood and the pulp and germinal centers are pretty generally infiltrated by red blood corpuscles. There is no localized collection of red cells suggesting hemorrhage but rather a generalized diapedesis of these cells. Large phagocytes containing brown granules and cell fragments resembling shrunken red cells are seen in great numbers. They are also spread diffusely throughout the spleen substance. The stroma seems to be very loosely placed and more open than usual suggesting moderate edema.

A large mesenteric *lymph node* shows marked hemorrhage into the node substance with separation of the node into small islands of cells. These are no longer compact but seem separated by edema. There are a few phagocytes containing brown granules but they are much less evident than in the spleen. The good condition of the red cells and the few phagocytes suggest that the hemorrhage is of recent occurrence.

A *rib* shows much hemorrhage in among the fat. In addition to the connective tissue framework are found occasional large round cells with vacuolated round nuclei. The cytoplasm stains faintly pink and the nucleus is not very dark and

cells rarely. When present they stain poorly and are pyknotic. Hemorrhage is diffuse.

In the *femur marrow* of each side, the fat and the framework are practically all that are visible. Only a few atypical darkly staining normoblasts remain.

The *end of the femur* shows a few more marrow cells but they stain darkly and frequently show chromatin granulation and pyknosis of the nuclei or they are very pale and partially autolyzed. Normoblasts are slightly more numerous. Hemorrhage is a prominent feature.

The following experiment shows the relative ineffectiveness of doubling the amount of radiation. The animal died on the 8th day instead of the 9th. Transfusion did not modify the intoxication nor the hemorrhagic tendency.

Dog 28-196.—A black and white adult female, well nourished, strong, and active. Weight 17.8 kg.

5.14.29. Hematocrit 53 per cent; plasma pale lemon color.

5.15.29. Condition and appetite excellent. Hematocrit 52 per cent; plasma pale lemon color. 8 p.m. Sodium amytal administered intraperitoneally. 1,080 m.m.m. with the usual factors were given over the upper and lower halves of the body as described except that both sides were treated with this amount making the total double the usual treatment or 2,160 m.m.m.

5.16.29. Condition good, able to walk but slightly ataxic.

5.17.29. Condition excellent. Appetite good. Hematocrit 50 per cent; plasma cloudy and very slightly pink. W.B.C. 10,900; 9,850.

5.18.29. Condition and appetite excellent. No diarrhea. Hematocrit 49 per cent; plasma lemon colored. W.B.C. 7,000; 6,700.

5.19.29. Condition good but some anorexia.

5.20.29. Condition good, though slightly inactive; anorexia. Hematocrit 44 per cent; plasma lemon colored. W.B.C. 600; 700.

5.21.29. Rather quiet; about $\frac{1}{3}$ of food eaten. Hematocrit 43 per cent; plasma pale lemon color. W.B.C. 200; 125. Weight 16.4 kg.

5.22.29. Weak and stuporous. No food eaten. Hematocrit 44 per cent, plasma lemon colored. W.B.C. 100; 100. Apparently somewhat dehydrated. Because of low W.B.C. the animal was transfused with 225 cc. of whole normal blood. Blood stained material was observed to pass from the rectum.

5.23.29. Moribund at 8:30 a.m. Death shortly after 10 a.m.

Autopsy, 2 p.m. The body is cold and rigor mortis present. The skin and mucous membranes are normal in appearance. The subcutaneous fat is somewhat reduced in amount. The musculature is good. The serous surfaces are all smooth, shiny, showing no hemorrhages. In the duodenum about 20 cm. below the pylorus is a segment about 10 cm. long, which is dark bluish red in color involving the mucosal, serosal, and muscular layers. The terminal ileum has a similar segment.

6.15.29. Nothing eaten—inactive—no changes noted. Mouth in good condition. W.B.C. 50; 50. Smear shows only 2 definite platelets in careful search of both cover slips. There are a very few white blood cells only 10 being found in the two cover slips, 6 being polymorphonuclear neutrophils, 1 disintegrated basophile, and 3 large mononuclears with kidney shaped or oval nuclei. There is considerable variation in size of the red cells, but no poikilocytosis. There is some achromia but this is variable since many of the cells have the usual amount of hemoglobin content. There is no stippling or basophilia. 1:30 p.m. Transfused with 200 cc. of citrated blood from same donor. 6:00 p.m. 200 cc. of Klim and Karo syrup given by stomach tube and immediately vomited. 11:00 p.m. No change—inactive and lethargic—not cold—does not look unusually sick.

6.16.29. Found dead though still warm this morning. No vomitus or stool in cage—no bleeding noted.

Autopsy, 11 a.m. The *body* is that of a poorly nourished female mongrel showing no gross lesions of the skin or mucous membranes. There is no evidence of bleeding in the mouth or rectum. There is no hemorrhage in the muscles nor is there any gross damage to the vascular system.

There are many ecchymotic areas in the parietal pleura especially in the left side of the chest, but no fluid or adhesions. There are several epicardial hemorrhages but none in the pericardial sac proper; no fluid or pericardial adhesions. The peritoneal surfaces are negative. The *heart* is essentially normal in size and shape and musculature shows no hemorrhages in its substance.

The *lungs* show very extensive rather solid hemorrhagic areas 1 to 2 cm. in diameter in sufficient number to occupy one-third of the total volume of the lung substance. The pulmonary vessels are free of thrombi.

There are two small and one fairly large sharply circumscribed bluish red areas in the mucosa of the lower ileum. The mucosa is thickened apparently by the infiltrating blood. The gastrointestinal tract except for postmortem autolysis is entirely normal. There is no gross evidence of injury from the radiation.

The *spleen* is normal in size—the architecture is undisturbed and shows no hemorrhage or congestion. Two of the *mesenteric lymph nodes* measure 1 x 1 x 1.5 cm. and are filled with blood. All other nodes here and elsewhere are small and pinkish in color, without gross hemorrhage.

The *bone marrow* of each femur and humerus contains abundant fat mixed with free blood suggesting hemorrhage. The rib and vertebral marrow are also hemorrhagic so that cellular details are obscured. The liver and other organs appear free of gross hemorrhage and are normal.

Microscopical sections are made from all tissues.

The *heart* shows some extravasation of blood into the epicardial fat and occasionally between the muscle bundles. There are no other abnormalities, the muscle cells being normal in appearance.

The bronchi of major lobules of the lungs are filled with blood. The alveoli are filled with blood cells, fibrin, and masses of bacteria—there are very few

shows generally a nucleolus. A normoblast is rarely seen. An occasional phagocyte containing brown granules is present. There is no evidence of any blood formation.

A thoracic *vertebra* shows much the same picture—there is a great deal of blood present between marrow fat cells. Aside from the connective tissue framework and a few phagocytes containing granules there are very few of the normal marrow cells to be found. There are a fair number of rather shrunken, abnormal looking normoblasts in each high power field, a few large round cells with pale cytoplasm and large round nucleus, and a few shrunken undifferentiated cells apparently of the myeloid series. The endosteum covering the bony trabeculae is normal in appearance.

The *femur marrow* shows marked hemorrhage, and a similar paucity of marrow cells. Cells resembling plasma cells and normoblasts without the pink cytoplasm are seen here and there in groups of two or three in the connective tissue framework.

Phagocytes with brown granules are about half as numerous as the marrow cells. Many areas of the section show only congested blood vessels and the connective tissue framework of the marrow.

The third experiment shows the characteristic course of events, unaffected by two transfusions, and death on the 9th day.

Dog 28-268.—A small black and white female mongrel. Somewhat thin, weight 8.6 kg.

6.5.29. Active. Hematocrit 46 per cent; plasma clear and normal.

6.6.29. Appetite good.

6.7.29. Lively, has gained weight—weight 9.1 kg. Hematocrit 46 per cent; plasma clear and normal. 8 p.m. Intravenous amytal—40 mg. per kg. Animal asleep in deep anesthesia before termination of injection. Deep therapy 1,050 M.A.M. were administered over the upper and lower halves of the body in two treatments, as described under Method.

6.8.29. Active—all food eaten, condition excellent.

6.9.29. Very active—appetite excellent. Hematocrit 43 per cent; plasma faint lemon color. W.B.C. 5,150; 5,000.

6.10.29. Active and playful—appetite good. Hematocrit 43 per cent; plasma faint pink color. W.B.C. 3,750; 4,450.

6.11.29. Active and playful—appetite good. W.B.C. 2,150; 1,900.

6.12.29. Condition good. Active and playful—appetite excellent. Hematocrit 39 per cent; plasma pale lemon color. W.B.C. 1,125; 1,125.

6.13.29. Most of food eaten—vomitus in cage. Somewhat less active than before. Mouth in good condition. No bleeding. Hematocrit 37 per cent. Plasma slightly pink. Weight 9.1 kg. W.B.C. 425; 350. 2 p.m. Transfused 100 cc. citrated whole normal blood from donor with hematocrit of 51 per cent—no reaction noted.

6.14.29. Inactive—nothing eaten, dog quiet and seems intoxicated. Hematocrit 41 per cent. Plasma lemon color. W.B.C. 75; 150.

DISCUSSION

The large *phagocytes* of the marrow, lymph glands, and spleen in these experiments present features of unusual interest relating especially to the origin of these phagocytes. We record above that these large phagocytes are numerous and contain red cells and other cell fragments. The lymphocytic cell chain is especially sensitive to radiation and all marrow cells are destroyed in satisfactory experiments. Yet these phagocytes persist and retain their usual capacity of engulfing much cell debris. It would follow from this observation that these particular phagocytes take their origin from the capillary endothelium which is obviously resistant to the radiation. This method may be a profitable one to study various types of phagocytes about whose origin there is some diversity of opinion.

The behavior of the blood platelets in this type of experiment calls for much more study but some points stand out in an analysis of these experiments. Platelets are present in the circulating blood up to the last 24 hours before death when they vanish. This platelet lack probably is in part responsible for the diffuse purpura found at autopsy. If the platelets were sensitive to radiation, we might expect an earlier decrease in the blood stream although not necessarily so. Assuming the probability that the megakaryocyte of the bone marrow produces the platelets, this observation suggests a life cycle of 7 to 8 days for the platelets in the circulation, as all megakaryocytes are destroyed by the marrow radiation.

SUMMARY

Constant findings were obtained in the acute reaction to the specified amount of heavily filtered radiation over the bony skeleton.

1. There develops without warning a short and fatal intoxication on the 8th or 9th day after the exposure to the radiation.

2. A profound leucopenia appears after 5 to 6 days and is maintained in the peripheral blood (200 white blood cells or less per c. mm.) for the 2 to 3 days before death.

3. The platelets suddenly disappear from the blood smears the day before death. This has some bearing on the life cycle of the platelet.

4. All of the organs and body structures present extensive and generalized capillary hemorrhage of recent origin.

wandering cells. Some alveolar walls are destroyed and cells disintegrated probably due to bacterial growth during the last day of life.

The *pancreas*, *adrenals*, and *kidney* aside from a moderate amount of capillary hemorrhage seem undisturbed in any way.

A section of *small intestine* through the abnormal area noted in gross shows extensive infiltration of the muscle, areolar tissue, and mucosa by red cells. Much of the epithelium of the villi has disappeared and that noted in the occasional villus is pyknotic and darkly stained. Hemorrhage into the submucosa has infiltrated and elevated the mucosa with the result that it is destroyed down to the muscularis mucosa. The pyknosis without destruction of the crypt nuclei indicates a moderate effect upon these cells directly by the radiation but the injury is insufficient to cause extensive destruction. In the *large intestine* a few localized hemorrhages in the mucosa are the only abnormal findings.

In the *spleen* the germinal centers are reduced in size and number. Their cells are darkly stained except for the numerous mitotic figures. The pulp cells are reduced in number but the pulp volume is increased by the red cells which are everywhere in large numbers. The red cells are well preserved and there are very few phagocytes present containing brown granules. There is no necrosis nor are pyknotic cells visible.

In the *mesenteric lymph node* the pulp is diffusely infiltrated by red cells, so much so as to separate the individual cells rather widely. The germinal centers are divided up into little islets of a few cells by the hemorrhage. The cells of the germinal center are not abnormal except perhaps that mitotic figures are quite numerous. A large number of huge phagocytic cells are everywhere and contain brown granules and whole red cells.

The central shaft marrow from the *humerus* and *femur* shows a few normoblasts here and there in the connective tissue framework supporting the fat and blood vessels. There are occasional phagocytes, containing brown granules. Small localized hemorrhages are everywhere. The usual number of marrow cells is greatly decreased. The diaphysis of the femur shows similar hemorrhage though not limited by the connective tissue framework. The marrow cells are greatly reduced in number yet are still quite numerous. Many "shadows" of marrow cells are visible. These cells remaining are very densely stained with hematoxylin and show shrunken coalescing chromatin material in the nuclei. The normoblasts and the cells of the same size and appearance except for the unstained cytoplasm show less disturbance than the more undifferentiated cells. There are a few phagocytes containing brown granules.

The *8th rib* on the right shows no marrow cells whatever, merely hemorrhage and the connective tissue and blood vessel framework. This side of the thorax was the side radiated. The *8th rib* on the left shows extensive hemorrhage, a rare normoblast, the framework cells. No other marrow cells are to be found.

The cervical, thoracic, and lumbar vertebrae all show similar depletion of the marrow cells with only an occasional normoblast present. Hemorrhage is everywhere, filling the spaces among the bony trabeculae.

5. The substance of the spleen and lymph nodes is greatly reduced and the germinal centers are visible only as remnants.

6. The red cell hematocrit reading drops from about 50 per cent or normal to approximately 40 per cent.

7. The bone marrow is depleted of all its cells except the connective tissue and fat cells, blood vessel endothelium, phagocytes filled with brown granules, and occasional normoblasts.

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active hyperplastic marrow which is endeavoring to replace the destroyed red blood cells. The effect of a destructive agent such as the heavily filtered radiation upon such a marrow is of great interest since the more actively growing and dividing cells should be more sensitive to radiation than the undisturbed marrow. On the other hand, a hyperplastic marrow by its increased volume alone might be more able to survive the destructive effects of the radiation. Each of the two agents seemed to be equally effective and their combination resulted in a shortening of the period before exitus without an increase in the destructive changes characteristic of each.

Method

Full-grown healthy stock dogs were used throughout. They were kept on a general diet and were observed for 2 weeks previous to being used, to eliminate the possibility of distemper. The colloidal silver preparation used was collargolum which was administered intravenously in a 1 per cent emulsion, as described above (Paper I). The dosage varied in the different animals. In most cases, one or more courses of collargol had been injected before the radiation was used. In one case, the silver was begun 2 days after the exposure to radiation.

The radiation was administered with the animal under amytal anesthesia (intravenous), since complete absence of motion was essential. The fully anesthetized animals were placed upon the deep therapy treatment couch, and the upper half of the body including the head and upper legs down to the costal margin was irradiated. The animal was shifted and the lower half of the body including the tail and lower legs irradiated. It is necessary to do this from the side of the animal so that the intestines can be protected from the radiation while the lumbar spine is exposed. This technique is described more in detail in Paper II in this series. The factors are 200 kilovolts, current 35 milliamperes, target skin distance 58 cm., filtration: copper 0.5 mm., aluminum 1.0 mm. Dosage varied from 350 to 1,050 milliampere minutes.

Blood was obtained from the jugular vein at the proper intervals for the hematocrit, white blood cell count, and blood smears. The latter were stained with Wright's stain. Complete autopsies were performed as soon after death as possible, the tissues being fixed in Zenker's fluid and stained with hematoxylin-eosin.

EXPERIMENTAL OBSERVATIONS

The following experiment illustrates the acute lethal effect from sublethal amounts of silver and heavily filtered radiation, the silver being given first, the hemolysis, anemia, leucocytosis, and loss of weight being characteristic of the silver effect. The precipitate drop

III. THE COMBINED EFFECTS OF COLLOIDAL SILVER AND HIGHLY FILTERED ROENTGEN RADIATION UPON THE HEMATOPOIETIC SYSTEM IN DOGS

By SAMUEL S. SHOUSE, M.D., AND STAFFORD L. WARREN, M.D.

*(From the Departments of Pathology and Radiology of the University of Rochester
School of Medicine and Dentistry, Rochester, N. Y.)*

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The production of an anemia in dogs by the repeated intravenous injection of colloidal silver has been described in a preceding paper (Paper I). This caused a severe anemia associated with peripheral blood destruction followed by bone marrow hyperplasia. The intoxication was accompanied by anorexia, lassitude, marked loss of weight, and resulted in death. The number of circulating leucocytes remained essentially normal or became elevated, and there were no signs of a platelet deficiency. Apparently the animals died from the generalized toxic effect of the silver, rather than from the anemia since the hematocrit level was distinctly compatible with life. In Paper II we described the acute changes following exposure of the whole bony skeleton to heavily filtered Roentgen radiation. This is characterized by a leucopenia, an anemia, absence of platelets, bleeding, and destruction of the bone marrow cells. Exitus occurs about 9 days after the radiation.

It was thought that the leucopenia and the failure of the platelets after exposure to radiation were intimately concerned with the death of the animal. Since colloidal silver injections commonly produced a leucocytosis and had no influence upon the platelets, injections of silver might conceivably have some influence in prolonging the life of the animal which had received a fatal amount of radiation over the bone marrow. The reverse was found to be true, however. The acute anemia in the radiated animal comes on at the same time that the hemorrhages occur and seems definitely related to them and not to any disturbance of the supply of red blood cells from the bone marrow. The anemia produced by the silver seems to be accompanied by an

days inclusive, in daily doses of 75 mg. to 135 mg., making a total of 650 mg. in 7 days. The leucocyte count was 7,850 cells on the 4th day and 275 on the 9th. On the latter date, the animal's general condition was good except for slight inactivity, but the following morning (10th day) she was moribund and exitus occurred before blood specimens could be obtained. No purpura or hemolysis had been observed. The initial hematocrit of 57 per cent had decreased to 53 per cent on the 3rd day, before the collargol was begun, and there was a steady decline to 33.3 per cent on the day before death. The appetite remained good, and there was practically no loss of weight.

Autopsy revealed extensive fresh hemorrhage in the lower ileum, and a few areas of hemorrhage in the bladder wall. Some of the mesenteric lymph nodes had their sinuses filled with erythrocytes, and contained many large phagocytes packed with red blood cells. The spleen showed many large mononuclear cells containing coarsely granular golden brown pigment, and some of them had also ingested red blood cells. The Kupffer cells of the liver contained a similar pigment. The bone marrow from all regions showed only a few marrow cells.

Although the sequence of events differs somewhat, these two experiments show the characteristics of both silver and radiation effects described in the first two papers of this series.

The following rather long experiment shows the effects of a moderate dosage of silver followed by a small dose of radiation. The silver effects (anemia) were maximal and the radiation effects slight and transitory. A transfusion and two more courses (moderate doses) of silver resulted in a marked leucocytosis. The anemia remained stationary for some time and then the hematocrit gradually rose. A large dose of radiation caused the usual leucopenia to below 100 cells with exitus before the development of the usual purpura seen after such a dose of radiation. Each agent thus demonstrated its characteristic influence in turn except that the destructive effects of the radiation upon the marrow cells and leucocytes was overwhelming in the end.

Dog 28-47.—A very strong male mongrel, weighing 27.6 kg., was given 100 mg. to 200 mg. of collargol daily during the first 11 days making a total of 1,875 mg. Conspicuous hemolysis was observed on the 14th day. The initial hematocrit of 53 per cent had declined to 30.4 per cent on the 17th day. That evening, the animal was given 350 m.A.M. of Roentgen radiation over the skeleton. Following that the leucocyte count dropped but did not fall below 7,000. The hematocrit was 25.7 per cent on the 25th day, but had risen to 29.2 per cent on the 29th. A transfusion was done that afternoon which raised the hematocrit to 43.8 per cent. The latter was 37 per cent on the 64th day. Then he was given 100 mg. of collargol

in the white blood cells and the paucity of cells in the bone marrow are typical radiation effects. The combined effects probably overwhelmed the animal so that exitus occurred too quickly for other radiation effects (purpura and hemorrhage) to appear.

Dog 28-158.—A very strong and active male mongrel weighing 21.6 kg. was given daily intravenous doses of 100 mg. to 157 mg. of collargol for 11 days, making a total of 1,467 mg. On the 21st day, he was given 1,050 m.a.m. of heavily filtered radiation over the bony skeleton from the left side. Prior to this the white blood cell count had been 16,000; 2 days after the radiation, it was 14,000, and the general condition was good. On the 4th day after the radiation the leucocyte count was 775; the animal seemed rather quiet, but presented no other abnormal signs. The following morning, he was moribund, with very deep respirations, and a leucocyte count of 250. No signs of hemorrhage were present.

While the silver was being administered and for a few days after it was stopped, he had anorexia, a loss of weight from 21.6 kg. to 17 kg., a leucocyte count of 16,400, definite hemolysis, and a decline of the hematocrit from 55 per cent to 32 per cent on the 21st day, just prior to the radiation. Following the latter, the hematocrit continued between 35 per cent and 32 per cent until the end, and there was no further hemolysis.

Autopsy revealed a few small hemorrhages in one lung, moderate hemorrhages in the mucosa of the large intestine, and considerable hemorrhage in the submucosa of the bladder. The liver showed a moderate degree of central congestion and atrophy with *golden brown material* in some of the endothelial cells. The spleen contained very numerous clumps of similar particles. The pancreas presented a small area of fat necrosis. The bone marrow from the right femur showed a slight increase in the number of marrow cells, among which there were several deposits of *golden brown particles*, (silver), but the marrow from all other regions contained very few cells.

The following experiment shows the acute lethal effect of sublethal doses of radiation and silver with the radiation administered first,—the reverse of the last experiment. The failure of the development of the leucocytosis would indicate that the depletion of the leucocytes by the radiation was generalized and not just restricted to the circulating leucocytes. The leucopenia to below 1,000 cells is usually associated with purpura and bleeding. The sections of some of the organs indicate that this was beginning.

Dog 28-153.—A very active and healthy female mongrel, weighing 17.5 kg. was given 700 m.a.m. of heavily filtered radiation over the skeleton from the left side. After this collargol was injected intravenously between the 3rd and 9th

Dog 28-200.—A vigorous male Airedale, weighing 20 kg., was given daily doses of 75 mg. to 150 mg. of collargol for 11 days, making a total of 1,375 mg. Near the end of this course of injections, the buffy layer in the hematocrit tube became about three times its original thickness. Hemolysis was observed between the 10th and 20th days and occasionally thereafter. The initial hematocrit of 46.8 per cent red blood cells had dropped to 27.1 per cent on the 17th day. Then it remained between 30 per cent and 35 per cent until the 121st day. On the 43rd day, he was given 700 M.A.M. heavily filtered over the bony skeleton from the left side. There was practically no change in the hematocrit but the leucocyte count declined from 16,000 to 3,750 on the 51st day (8 days after the irradiation). It was 3,900 on the 52nd day, but had risen to 6,000 on the 55th. It remained between 6,500 and 11,000 until the 71st day. That night, he was again radiated, this time with 1,050 M.A.M. over the right side. Following this, there was a gradual decrease in the number of white blood cells to 3,000 on the 82nd day (11 days after the irradiation), and then a slow rise to 12,000 on the 99th day. On the 102nd day, when the count was 10,200, he was again given 1,050 M.A.M. of heavily filtered radiation over the skeleton from the left side. The leucocytes declined to 1,100 on the 109th day (7 days after the irradiation), but gradually returned to 8,000 on the 128th day, and were 7,000 on the day of exitus. Although the hematocrit had not been affected by the previous exposures to Roentgen radiation, having maintained a value of 30 per cent to 35 per cent since before the first radiation, yet on the 121st day, it dropped to 29 per cent, and gradually declined to 19 per cent on the 129th day. That afternoon, he was transfused with the calculated amount of blood to raise his hematocrit to 30 per cent. The following day, it was down to 22 per cent, and he was transfused again. He was found dead the next morning, the 131st day of the study, and the 29th since the last exposure to the radiation. Prior to the transfusions, the blood smears showed very marked achromia and anisocytosis, a few normoblasts and basophilic erythrocytes, a very few myelocytes, but no lymphocytes. Although only a very few platelets were seen in the smears, no hemorrhages were observed during life.

The initial weight of 20.2 kg. fell to 18.3 kg. after the course of collargol and before the radiation was employed, and down to 16.4 kg. near the end. He showed lethargy and anorexia during the last 13 days, and was gavaged with milk and glucose. Pitting edema of the legs was present the last 10 days.

Autopsy showed emaciation, extensive pitting edema of the legs, and 500 cc. of thin yellowish fluid in each pleural cavity. The lungs presented extreme edema and congestion, and microscopic study revealed some regions with many red blood cells in the alveoli. There was moderate congestion of the gastrointestinal tract. The liver showed gross scarring along the margin of some of the lobes. Slides from these regions showed extensive fibrosis, in which occurred remnants of the liver columns and numerous large mononuclear cells filled with coarsely granular golden brown pigment. In other sections, the Kupffer cells were also filled with similar pigment which, however, had a greenish blue color when stained for iron. The spleen contained many normoblasts and a few small scattered deposits of coarsely

for 3 days, and again from the 70th until the 77th day, making a total of 2,975 mg. As a result of this, the leucocyte count rose as high as 26,000 although the hematocrit was only 25 per cent between the 80th and 90th days; but it gradually rose to 35 per cent on the 103rd day.

That night (103rd), the animal was radiated with 1,050 m.a.m. over the skeleton. The leucocyte count remained as high as 13,000 for 2 days (until the 105th day), but the next morning it was 6,150. On the 108th day, there were only 425; on the 109th, 225; and on the 110th day, 100 leucocytes per c. mm. Previously his general condition had remained fairly good, but on that day (110th), he was definitely weak and lethargic; however, no antemortem hemorrhages were observed. He died that night, 7 days after the last radiation. The hematocrit was 31 per cent on the day of death. A postmortem blood examination showed no leucocytes in two counting chambers. The initial weight of 27.6 kg. had declined to 22 kg. prior to the last "x-ray" exposure, and terminally, it dropped to 19.5 kg.

Autopsy revealed numerous small hemorrhages in the parietal and visceral peritoneum. The intestinal lumen contained very abundant clotted blood, and in several loops, the entire thickness of the wall was filled with hemorrhage. The spleen had a dark slaty color, and microscopical study disclosed numerous deposits of coarsely granular golden brown particles. The liver presented a slight excess of connective tissue radiating from the portal spaces, and many large deposits of coarsely granular golden brown particles. The pancreas had a slaty gray color, and contained numerous small deposits of granules similar to those in the liver and spleen. The bone marrow in the shaft of each femur and each humerus had a very dark bluish red color and a very soft consistency. Microscopic study revealed engorged sinuses, abundant fat, and very few marrow cells. There were many deposits of coarsely granular golden brown particles, both intra- and extracellular, and also many large phagocytes containing red blood cells. The marrow from the cervical, thoracic, and lumbar vertebrae contained abundant fat and dilated congested blood vessels. Only a very few marrow cells were present. Here, there were also a few intracellular deposits of coarsely granular brown particles. Likewise, the marrow from the second and the eighth ribs on each side contained only a few marrow cells and was congested.

The following long experiment shows the characteristic acute and chronic effects of the silver and radiation, the anemia being especially marked. Repeated transfusion near the end of the experiment did not change the course of the experiment. The second and third doses of radiation should have caused exitus on the 9th or 10th day. The survival of this animal after the second radiation and the long period after the third radiation can be explained only by the failure to include some of the bone marrow in the field of radiation each time. The presence of silver in the tissues does not seem to change the effect of the radiation any, i.e. by intensive local radiation.

transfusion, but unfortunately no protein determinations were done. The serum non-protein nitrogen was taken in one of the animals. It remained normal until a very few days before death, and then became moderately elevated.

In the shorter experiments, the anemia resulting from the silver injections was not further affected by the radiation but remained the same until death occurred. In the last experiment described above, the hematocrit was not affected by any of the radiation until 10 days before death. Then it declined very rapidly, and could not be maintained by repeated transfusions. Since this animal withstood the first two exposures to the radiation and survived an unusually long time after the third, it seemed probable that not all of the bones had been completely radiated. This was borne out by the finding of a few islands of hyperplasia in one femur.

In most of the experiments, the autopsy revealed either brown granular pigment or phagocytosed red blood cells in the various organs. We can not say positively whether the hemophage activity was stimulated by the colloidal silver or whether this represented a sieving out process of injured erythrocytes. The amount present did not seem to be increased over that found after the use of silver or radiation alone. Along with these signs of peripheral blood destruction, there was an extensive aplasia of the bone marrow. Blood smears showed marked achromia and many young forms of red blood cells. These young red blood cells probably represent a desperate attempt of the remaining islands of bone marrow cells to regenerate the blood. Mortland reported similar changes in several human cases of thorium poisoning.

Even though all of these animals had a severe anemia and depletion of the bone marrow, death was not apparently the result of an insufficient oxygen carrying capacity of the blood but in some way was caused by a deficiency of thrombocytes and leucocytes. There might have been some unknown irreparable injury to the fixed tissues, especially the platelet forming mechanism, since life could not be saved by repeated transfusions. The actual effect of this on the body is not clear, since the hemorrhages *per se* were entirely too small to be fatal and no infection was superimposed on the agranulocytic condition. The toxic effect of the phagocytized silver is not to be for-

granular golden yellow pigment, both intra- and extracellular, which was colored greenish blue with the iron stain. The retroperitoneal lymph nodes showed extensive clumps of similar pigment in the stroma, the sinuses filled with erythrocytes, and many large mononuclear cells with ingested red blood cells. Some of the germinal centers showed islands of activity, but the most of them seemed to be destroyed. The bone marrow was congested and showed a few areas of hemorrhage. There was a marked loss of the normal elements; but several islands of densely packed cells remained which consisted chiefly of normoblasts and some myeloid cells.

DISCUSSION

BOSTON PSYCHOPATHIC HOSPITAL

The combined effects of silver and radiation did not differ much from what would be expected from the effects of each alone. Those animals which were given a course of silver injections several days prior to the irradiation showed toxic signs such as anorexia, loss of weight, leucocytosis, definite hemolysis, and a severe anemia. All of these symptoms had improved considerably before the Roentgen radiation was given. Abundant silver particles were found in the reticulo-endothelial cells. The less acute experiments showed scarring in the liver in which there were numerous deposits of silver. Thus the action of the silver here was similar to its effect when used alone.

Following the exposures to moderate amounts of heavily filtered Roentgen radiation, there was a marked decrease in the number of leucocytes, which almost completely disappeared within 5 to 10 days. Exitus occurred soon after this. It is remarkable that most of the animals remained in apparently good condition, becoming lethargic only a few hours before death. The blood smears showed only a few platelets, yet none of the dogs presented any signs of purpura. Autopsy revealed rather definite depletion of the bone marrow in all of the animals, and also prominent hemorrhages in the viscera. No terminal infections were observed.

It is interesting that one experimental animal showed a definite leucopenia after each of his three exposures to radiation, but had a leucocyte count of 7,000 when death occurred, 29 days after the last radiation.

Edema which occurred in an occasional animal in the longer experiments, was thought to be the result of malnutrition and a consequent lowering of the plasma proteins, since it was improved slightly by a

gotten as a direct cause of death with the animal's general condition so much depressed.

CONCLUSION

1. The individual destructive effects of colloidal silver and heavily filtered radiation are still evident when the two are used together.
2. The combined effects are cumulative in that small doses are more destructive than when either is used alone.
3. The leucocytosis resulting from the injection of the colloidal silver affords no protection against the terminal leucopenia following the radiation.

remained sterile at the end of 7 days. However, in anaerobic cultures prepared according to the Smith-Noguchi technique, minute spherical bodies were observed which were considered to be organisms and which in the first subculture generation proved infective, producing colds in ten out of eleven inoculated volunteers. In conclusion, Foster stated that he believed that the infectious agent of the common cold was a filtrable virus and that by utilizing a specialized culture medium, he was able to cultivate from filtrates containing active virus, a peculiar minute filter-passing microorganism which had a direct causal relation to colds.

In 1917 Dold (3) reported that he had been able to transmit an upper respiratory infection to human volunteers by means of a bacteria-free Berkefeld filtrate of the nasal secretions obtained from an individual ill with a natural "cold." The nasal secretions were diluted in fifteen volumes of physiological saline and then filtered through a Berkefeld candle under negative pressure. In the first group of experiments seventeen volunteers were inoculated with the Berkefeld filtrate. Fifteen students who worked, ate and slept in the same rooms with the volunteers served as controls. Seven of the inoculated volunteers (41 per cent) developed typical "colds" within 72 hours. None of the controls developed the affection during the period of observation. In a second experiment forty patients on the surgical wards were inoculated with a Berkefeld filtrate of the diluted nasal secretions from an individual ill with a "cold." The results were essentially negative as only one developed an upper respiratory infection. The filtrates were cultured in serum agar, ascitic agar and in ascitic broth under both aerobic and anaerobic conditions. Special cultures were made in a modified Smith-Noguchi medium in which ascitic broth replaced the usual ascitic fluid. The cultural results were negative in all instances. Diluted material from a 48 hour Smith-Noguchi culture of the filtrate of nasal secretions obtained from a volunteer in the first group who developed a "cold" subsequent to inoculation, was used in the third experiment. Two of these volunteers inoculated with this material developed mild upper respiratory symptoms which endured 4 or 5 days. The culture from which this material was obtained, was considered to be free of bacteria. In this paper experimental observations are also reported to the effect that, irritating substances (insect

THE ETIOLOGY OF ACUTE UPPER RESPIRATORY INFECTION (COMMON COLD)*

By PERRIN H. LONG, M.D., JAMES A. DOULL, M.D., JANET M. BOURN, Ph.D.,
AND EMILY McCOMB, Ph.D.

(From the Department of Medicine and the Clinical Laboratory of the John J. Abel
Fund for Research on the Common Cold, The Johns Hopkins
University, Baltimore)

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In 1914 Kruse (1) reported that he had produced colds in human volunteers by intranasal inoculations with Berkefeld filtrates of the nasal secretions from individuals ill with natural colds. The nasal secretions were diluted in fifteen volumes of physiological salt solution and filtered through a small Berkefeld candle. A few drops of this filtrate were instilled into the nostrils of twelve volunteers. After an incubation period of from 1 to 3 days, four of the twelve developed colds. Later in the same year the experiment was repeated and of the thirty-six individuals who were inoculated, fifteen developed colds with an incubation period varying from 1 to 4 days. In both instances it was not possible to demonstrate bacteria in the filtrates by means of aerobic or anaerobic cultures methods. These results led Kruse to conclude that the infectious agent of colds was a filtrable virus and to this virus he gave the name—*Aphanozoon coryzae*.

A short time after Kruse's note appeared, Foster (2), in a careful study confirmed and extended Kruse's observation. Foster obtained the nasal secretions from early acute colds and after diluting and shaking them in salt solution, the mixture was filtered through a tested Berkefeld N candle. Seven of the ten healthy young men inoculated intranasally with a few drops of the filtrate developed typical colds within 48 hours. Human blood-agar plates prepared from the filtrates and incubated at 37°C., under both aerobic and anaerobic conditions,

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instance the cultures remained sterile. Smears from the filtrates invariably showed coccoid bodies similar to those described by Foster. No significance was attached to these findings since the same bodies could be demonstrated in filtrates of the nasal washings from normal persons. "Inoculations with the filtrates were made into the nostrils of volunteers as soon after filtration as possible. In no case did this time exceed twenty-four hours after the collection of the secretions, which in the meantime were kept in the ice-box." One hundred human volunteers were inoculated with the various filtrates and five of them developed upper respiratory infections following the inoculations. Because of the small number of positive results, these observers considered them to be the result of causes entirely independent of the inoculations, and concluded that their experiments presented no convincing evidence indicative of a filtrable agent as being the exciting factor in acute coryza.

In an extensive study of acute upper respiratory infection, Dochez and his associates have investigated all of the possible etiological factors in this affection. Early in the course of their investigations (8) they demonstrated that the aerobic flora of the nasopharynx played little, if any, rôle in the production of infectious "colds." Later (9) in a study designed to ascertain the etiological significance of the anaerobic filter-passing flora of the nose and throat, similar conclusions were drawn in regards to anaerobic microorganisms. Recently, Dochez, Shibley and Mills (10) reported that in the course of their investigation on human upper respiratory infections, they were able to produce in apes, by means of inoculations with Berkefeld V filtrates of the nasopharyngeal washings from individuals ill with "colds," a condition which bore a striking resemblance to the human disease. In this communication they stated that as yet the active filtrable agent was unknown, but that from the filtrate in all positive experiments, a Gram-negative anaerobic bacillus of the type described by Olitsky and Gates, had been cultivated. In a further report (11) they stated that they had been unable to infect apes with the filtrates of nasopharyngeal washings from normal individuals, notwithstanding the fact that from 75 per cent of these filtrates, they cultivated Gram-negative anaerobes. These findings led them to believe that the type

powder) can when inhaled, produce the symptoms and signs of an acute infectious "cold."

During the influenza epidemics of 1919 and 1920, Schmidt (4) inoculated one hundred and ninety-six persons with Berkefeld filtrates of nasal secretions from colds and eighty-four individuals with Berkefeld filtrates of the respiratory tract secretions from individuals all with "grippe." In the first group, twenty-one developed colds and three developed grippe; while in the second group, five developed grippe and four colds. Of forty-three controls who had been inoculated with sterile physiological salt solutions eight developed colds. No information is given in this report as to the exact size of the Berkefeld filter or to the period in the disease when the secretions were collected. Schmidt in concluding his report, expresses the belief that more human beings must be inoculated before the true nature of the infectious agent is determined.

Shortly after this communication appeared, Williams, Nevens and Gurley (5) reported that they had been unable to infect forty-five volunteers with Berkefeld N filtrates of the nasopharyngeal washings from seven early "cold" cases and three typical influenza cases.

During investigations concerning the etiology of influenza, Olitsky and McCartney (6) studied the filtered nasopharyngeal secretions of numerous individuals, ill with "colds." They were unable to produce any characteristic lesions in rabbits, by intratracheal inoculation with Berkefeld V or N filtrates of the nasopharyngeal washings from these subjects. However, they were able to cultivate several groups of minute anaerobic Gram-negative organisms from some of these filtrates. In their opinion, none of these minute organisms were constant enough to be considered the incitant of the affection. By using as the inoculum, filtered nasopharyngeal secretions from early cases of typical infections, they were able to transmit singly and in series upper respiratory infections to human volunteers, thus demonstrating that the incitant was a filtrable agent.

Robertson and Groves (7) collected the nasal secretions of persons ill with uncomplicated coryza from 6 to 144 hours after the onset of the infection. After diluting these secretions with sterile salt solution, the mixtures were filtered through Berkefeld candles. The filtrates were cultured upon aerobic and in anaerobic media and in every

conclusions of Dochez and his associates that the contagious "cold" is caused by a filtrable agent which probably belongs to the group of so called filtrable viruses.

Methods

Healthy, intelligent young women were selected as volunteers for the test. A careful history, in which special emphasis was placed upon previous upper respiratory infection, was taken in each instance. The nose and throat of each subject was examined* and roentgenograms of the sinuses and chest were taken before accepting the applicant. The first twelve tests were performed upon the volunteers who were kept in single rooms on the isolation ward. There were no other patients in this ward during the experimental periods. In the second group of eight tests, in order to avoid having subjects with upper respiratory infection on the same pavilion in which other subjects were passing through the required control period, the volunteers were placed in pairs in suites of rooms on the private wards and on the isolation ward. This plan ruled out the possibility of accidental infection through direct contact, and allowed us to utilize the patients on the private wards as a control group. It also permitted test transfers to widely separated groups of individuals.

The volunteers were kept in strict quarantine. The nursing care was performed by graduate nurses. Only one observer (P. H. L.) came into contact with the subjects until symptoms had developed. Hands were scrubbed and sterile masks and gowns donned before coming into contact with the individual volunteer. All eating utensils were sterilized. The nurses and the observer were examined daily for upper respiratory infection.

Each subject had a thorough physical examination upon entry. White blood cell counts were taken daily and the urine was examined frequently. Observations of the temperature, pulse and respirations were made at 4 hour intervals during the control and incubation periods, and at two hour intervals after the onset of symptoms.

Daily aerobic cultures of the tonsils and posterior pharyngeal wall were examined for the presence of *H. influenzae* and *Streptococcus haemolyticus*, type Beta. In studying *H. influenzae* the sodium oleate hemoglobin agar plates (pH 7.4) described by Avery (15) were employed. The technique of isolation and identification of *H. influenzae* on these plates has already been described by Bourn (16) in her studies of these organisms.

The examinations for the presence of *Streptococcus haemolyticus*, type Beta, were made by placing the nasopharyngeal swab in beef infusion broth (pH 7.4-7.6) and allowing it to incubate for at least 2 hours at room temperature. Dilutions of the two-hour cultures were inoculated into liquid 5 per cent whole rabbits' blood

* The nose and throat examinations were performed by either Dr. J. J. Chisolm or Dr. S. W. Egerton of the Department of Otolaryngology.

of upper respiratory infection under consideration was caused by a filtrable virus.

In two recent reports Dochez and his associates (12, 13) describe the results of their transmission experiments in apes and in human beings. Of twenty-eight ape experiments concerned directly with the testing of the hypothesis that colds may be caused by filtrable agents, washings from individuals with colds were used in twenty instances and normal washings in eight. Four animals in the first group were excluded for statistical purposes. Seven of sixteen animals contracted colds subsequent to inoculation with filtered nasal washings from individuals ill with natural colds. In the group of eight animals inoculated with filtrates of nasal washings from normals, none developed colds, although 75 per cent of these filtrates contained Gram-negative filter-passing microorganisms. Several miscellaneous experiments were performed including two successful ape to ape transmissions in which filtered ape washings were used and two unsuccessful experiments in which an attempt was made to transfer colds by means of living cultures of the filter-passing anaerobes obtained from a filtrate which had been used in a successful transmission experiment. In the course of their experimental transmission tests in apes several positive results were obtained with filtrates in which the filter-passing anaerobes were absent, thus substantiating their view that the filter-passing anaerobes were not the primary etiological agents in the production of "colds."

The human transmission tests were carried out under a most rigorous system of quarantine in which every precaution was taken to protect the subjects from direct or indirect contact with natural colds. Nine completed experiments are reported in which transmission was attempted by means of filtered nasal washings and of these four were successful. As a result of their experimental observations these investigators conclude that "the contagious cold in human beings is caused by an invisible, uncultivable, filtrable agent which in all likelihood belongs to the group of so-called submicroscopic virus."

The observations to be recorded in this paper have already been briefly referred to in a short report (14). They were undertaken at a time when the most recent communications of Dochez and his associates were in the course of publication. As will be seen from the text, our observations confirm their experimental work and support the

pharynx and nasal passages was considered necessary for the diagnosis of "a cold" in the inoculated individual.

Transmission Tests in Man

Protocols of Individuals Ill with Natural "Colds."—

Patient A.—No history of exposure. 6/11/30—Upon awakening, her head ached and her throat was sore. A thin watery nasal discharge and intermittent nasal obstruction were present. The nose and throat examination was essentially normal except for a small amount of nasal discharge. 6/12/30—Quite uncomfortable. Marked constitutional symptoms were present, and nasal discharge and obstruction more marked. The cold endured 5 days. No aerobic cultures were made. Anaerobic cultures of the Berkefeld V filtrate showed Group III A and a diphtheroid. Cultures of the Seitz filtrates were negative.

Patient B.—6/12/30—At 4 a.m., developed sneezing, irritation of the throat, and a profuse watery nasal discharge. Soon this became more profuse and nasal obstruction and profuse watery discharge. Examination: Skin of anterior nares was reddened and appeared to be irritated by the nasal discharge. Mucous membranes of the turbinates and septum were very red. Moderate amount of thin watery discharge seen in both nostrils. Tonsils small, the left tonsil inflamed; the posterior pharyngeal wall hyperemic. Aerobic cultures negative for *Streptococcus haemolyticus*, type Beta, and *H. influenzae*. Anaerobic cultures of the Berkefeld V filtrates showed Group IV, Group III A and an unidentified rod. Cultures of the Seitz filtrate were negative.

Patient C.—No known exposure. 6/11/30—Fatigue, drowsiness and smarting of the eyes. 6/12/30—On awakening, felt very tired; throat was sore, back ached and a thin watery nasal discharge with nasal obstruction was present. Examination showed slight swelling and hyperemia of the inferior turbinates on both sides. A small amount of mucoid nasal discharge present. The tonsils hyperemic and injected. Aerobic culture showed no hemolytic streptococci, type Beta, or *H. influenzae*. Anaerobic cultures and cultures of the Seitz filtrate negative. This infection endured for 5 days.

Patient D.—6/17/30—During the afternoon of 6/13/30 was exposed to an early cold. During the night of 6/15/30 developed sneezing and a sense of dryness and fullness in throat. On the following day throat was quite sore and intermittent nasal obstruction and a scant thin watery nasal discharge appeared; generally uncomfortable. Symptoms increased in intensity and by the next day he was in addition suffering from headache and slight malaise. Examination: Temperature 99°F. (by mouth). Mucous membranes of nose slightly injected. Good breathing space present on the right; on the left the inferior turbinates much enlarged. Practically no discharge was present on either side. Tonsils small; the mucous membranes of the posterior pharyngeal wall bright red. No evidence of sinus infection. The duration of affection was 7 days. Aerobic cultures negative for *Streptococcus haemolyticus*, type Beta, and *H. influenzae*. Anaerobic cultures of the Berkefeld

agar, and plates were poured. After 24 hours incubation at 37°C. these plates were examined for the presence of hemolytic colonies and identification was completed by fishing individual colonies for smear preparations.

During the control period and after inoculations, nasopharyngeal swabs were examined for the presence of *D. pneumoniae*. These swabs were suspended in whole rabbits' blood broth and were incubated at room temperature for several hours. Then 1 cc. of the blood broth suspension was injected into the peritoneum of a white mouse and whole rabbits' blood agar plates were streaked with material from the suspension. The usual white mouse technique for the determination of pneumococci was followed and pneumococcus-like organisms were tested for bile solubility and typed serologically by means of Groups I, II and III specific anti-pneumococcus serum. The anaerobic filter-passing flora of the nose was studied at entry and further studies of these organisms were made after inoculation. The material for the anaerobic studies and for inoculation purposes was obtained by running 10 to 15 cc. of warm yeast infusion broth (pH 7.6) through the nasal passages and back into the nasopharynx. The washings were collected in a sterile bottle, shaken vigorously with glass beads and filtered either through tested Berkefeld V or W candles or through a small Seitz filter (Uhlenhuth model) (17). Cultivation of the filtrates was carried out in Smith-Noguchi medium (Olitsky and Gates modification), in yeast infusion broth (pH 7.6), and upon 5 per cent rabbits' blood dextrose yeast infusion agar plates. All cultures were incubated 10 to 14 days before being discarded.

The subjects with natural colds, from whom the inoculum was obtained, were carefully selected. The type of infection was investigated in an effort to rule out hay fever and acute sinus infections. The inoculum was obtained in each instance during the early hours of the affection. Regional aerobic cultures were planted and the nasopharyngeal washings were quickly passed through the desired filters. Not more than $\frac{1}{2}$ hour elapsed from the time of the washing until the subjects were inoculated.

The volunteers were observed during a control period of from 2 to 6 days (generally 5 days) prior to inoculation. During this time they received from two to five nasal instillations of filtered yeast infusion broth which were given in the same manner as the inoculations and were designed as a check upon the psychological reactions of the subjects and also to prevent them from knowing the time of inoculation. The technique of inoculation was as follows: the volunteer was placed in a supine position with the head tipped back and from 0.5 cc. to 1.0 cc. of the filtrate was introduced into each nostril. Then the mucous membranes of the anterior nasal passages and the posterior pharyngeal wall were lightly rubbed with swabs soaked in the filtrate. Each individual received from one to three inoculations within a period of 24 hours.

As soon as a volunteer complained of symptoms, careful examinations of the nose and throat were made by several observers. These examinations were repeated at frequent intervals during the infection. Detailed records were kept of all symptomatic and objective findings. The presence of objective changes in the naso-

and cough had made their appearance, with a slight frontal headache. Examination: Temperature 98.6°F. (by mouth). Good breathing space on both sides. No definite changes in mucous membranes of nasal passages. A small amount of watery discharge on floor of each nostril. Five hemorrhagic spots on buccal mucous membranes. The pharynx slightly injected. No aerobic cultures were made.

Anaerobic cultures of the Berkefeld V filtrate showed Group XVII organisms. Culture of the Seitz filtrate negative.

Patient I.—No history of exposure 7/12/30. While in a motor during the evening noticed a slight watery anterior nasal discharge. Did not sleep well and on the next day developed a frontal headache, sneezing, anterior and posterior nasal discharge, intermittent nasal obstruction and a sore throat. On 7/14/30 these had increased in intensity and hoarseness, a cough, and a small amount of mucopurulent sputum were present. Examination: Temperature 99.4°F. (by mouth). The mucous membrane of the turbinates swollen and injected. Poor breathing space on both sides. Very little nasal discharge seen. The entire pharynx moderately hyperemic. No aerobic cultures were taken. Anaerobic cultures of Berkefeld V filtrate and cultures of Seitz filtrate negative.

Protocols of Transmission Tests.—The material for the inoculation of volunteers was procured from the patients whose clinical histories have just been given.

Volunteer I.—Age 20. Influenza in March, 1929. One "cold" during the past 2 years. Physical and nose and throat examinations normal. Five-day control period. Inoculated on 6/11/30 with Berkefeld V filtrate from Subject A and on 6/13/30 with a mixed Berkefeld V filtrate from Subjects B and C. No symptoms or signs of an upper respiratory infection developed; discharged on 6/16/30. On the 1st day of the control period Type IV pneumococci in cultures from the nasopharynx. Subsequent daily cultures negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae*. Anaerobic cultures negative. White blood cell counts and the urine revealed normal findings at all times.

Volunteer II.—Age 22 years. Influenza in 1918. One cold during the past 2 years. Physical and nose and throat examinations normal except for moderately hypertrophied tonsils and adenoids. Five-day control period. Inoculated on 6/11/30 with a Seitz filtrate of nasopharyngeal washings from Subject A and 6/11/30 with the mixed similar filtrates from Subjects B and C. 6/13/30—Slight fullness and dryness in throat and a slight anterior nasal discharge, which disappeared within a few hours; discharged on 6/17/30. During the first 4 days of control period and on the 3rd and 5th days after inoculation, showed non-hemolytic *H. influenzae* in cultures from nasopharynx. Hemolytic *H. influenzae* present on the 3rd day after inoculation. No hemolytic streptococci, type Beta, or *D. pneumoniae* found. Anaerobic cultures taken before inoculation showed no growth, while from similar cultures taken after inoculation Group III A and XVII organisms were isolated. White blood cell counts and the urine were normal at all times.

filtrates showed Groups IX, III A, and IV. Cultures of the Seitz filtrates remained sterile.

Patient E.—6/24/30—No history of previous exposure. During the evening of 6/22/30 noticed dryness and fullness of the throat which by the next morning had developed into mild sore throat, accompanied by sensations of warmth and cold, generalized aching and nasal discharge and obstruction. On the next day the same signs and symptoms persisted, with, in addition, hoarseness and cough. Examination: Temperature 99.2 (by mouth). Fair breathing space on both sides. The nasal mucous membranes slightly swollen and hyperemic. A small amount of thin watery discharge present. Tonsils very small and not inflamed. The posterior pharyngeal wall was injected and a slight amount of mucoid posterior nasal discharge was seen. The glands at the angles of the jaw were enlarged and tender. Aerobic cultures were negative for *Streptococcus haemolyticus*, type Beta, and *H. influenzae*. Anaerobic cultures of the Berkefeld V filtrate showed two unidentified organisms. Cultures of the Seitz filtrates remained sterile.

Patient F.—6/25/30—No known exposure. During the afternoon of 6/24/30 noticed a sensation of fullness, dryness and of irritation of the throat with chilliness, aching of the eyes, slight nasal discharge and slight nasal obstruction. After a restless night, throat was sore, eyes ached, nasal passages were irritated, with marked increase in the amount of nasal discharge and obstruction. There was slight malaise, and by afternoon hoarseness. Temperature 99.4°F. (by mouth). Good breathing space in both nostrils. Moderate hyperemia and slight swelling of the mucous membrane, with a small amount of watery discharge, found in the nasal passages. The pharynx and nasopharynx moderately hyperemic. Aerobic cultures negative for hemolytic streptococci, type Beta, and *H. influenzae*. Anaerobic cultures of the Berkefeld V filtrate showed Groups IV and III A. Cultures of the Seitz filtrates were negative.

Patient G.—6/25/30—No known exposure. During the morning of 6/24/30 developed a sensation of dryness, fullness and irritation of the throat. In a short time thin nasal discharge, nasal obstruction and lacrimation appeared. On the following day, an increase in the intensity of signs and symptoms plus a feeling of moderate malaise. Examination: Temperature 99.4°F. (by mouth). Good breathing space on the right. Turbinates and mucous membranes on right seemed normal. Poor breathing space on the left side due to quite marked deflection of septum and to hyperemia and swelling of the mucous membranes. Small amount of mucoid nasal discharge. The pharynx was moderately injected. Aerobic cultures negative for *Streptococcus haemolyticus*, type Beta, and *H. influenzae*. Anaerobic cultures of the Berkefeld V filtrate showed Group IV. Seitz filtrate yielded sterile cultures.

Patient H.—6/26/30—History of exposure to a draught during 6/25/30. Early in the morning of 6/26/30 developed a sensation of fullness, dryness and irritation in the throat, slight nasal discharge and intermittent nasal obstruction. On the following day these had increased in intensity and lacrimation, hoarseness

amount of mucopurulent discharge on the floor of each nostril. The entire pharynx was slightly injected. 6/22/30—The nasal discharge and obstruction persisted although the subject felt much better. Temperature 99.2°F. (by mouth). 6/23/30—Marked decrease in the discharge and obstruction. 6/24/30—Very little nasal discharge. Crusting in both nostrils. Slight nasal obstruction. Volunteer was discharged on this day. Aerobic cultures from the nasopharynx were negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. Anaerobic cultures showed Group IV organisms both before and after inoculation. The white blood cell counts and urine were normal throughout.

Volunteer VII.—Age 21 years. Tonsillectomy and adenoidectomy in 1918. Two colds during the past 2 years. Physical and nose and throat examinations normal, except for a deflected septum. Six-day control period. Inoculated on 6/24/30 with a Berkefeld V filtrate of nasopharyngeal washings from Patient E. On 6/26/30, 50 hours after this inoculation, a sensation of fullness and irritation in throat, sneezing and of a thin watery nasal discharge. Nose and throat examination: slight diffuse hyperemia of nasal and pharyngeal mucous membranes and a small amount of thin watery nasal discharge. 6/27/30—Nasal discharge while profuse was quite intermittent as was also nasal obstruction. Moderate hoarseness and coughing. Nose and throat examination showed only slight changes from normal. Volunteer forced to leave the hospital on 6/27/30 and was followed by telephonic reports. On 6/28/30 nasal obstruction and discharge present. Temperature 99.2°F. (by mouth). Hoarseness had decreased in severity. 6/29/30—Hoarseness and coughing persisted. Intermittent nasal discharge. 6/30/30—Intermittent nasal discharge and obstruction, with crusting. 7/1/30—Nasal discharge was scant and crusting had increased. 7/2/30—Nose practically normal. Non-hemolytic *H. influenzae* isolated in cultures from the nasopharynx on the 1st and 5th days of the control period. *D. pneumoniae*, hemolytic streptococci, type Beta, and hemolytic *H. influenzae* were not isolated in any cultures from the nasopharynx. Anaerobic cultures taken in the control period were negative while those obtained on the 3rd day after inoculation showed Group III A organisms. White blood cell counts and the urine were normal throughout.

Volunteer VIII.—Age 22 years. Tonsillectomy and adenoidectomy in 1925. One cold during the past 2 years. Physical and nose and throat examinations normal. Six-day control period. Inoculated on 6/24/30 with the Seitz filtrate of nasopharyngeal washings from Patient E. 24 hours after this, a sensation of fullness, dryness and of irritation in the throat, slight nasal obstruction and a slight amount of thin watery nasal discharge; felt quite listless and drowsy. Did not sleep well. 6/26/30—Moderate mucoid anterior and posterior nasal discharge, nasal obstruction, headache, mild malaise, soreness in the nasal passages and throat, and a temperature of 99.4°F. (by mouth). Nose and throat examination: Good breathing space on both sides. Inferior turbinate on right swollen and hyperemic. A small amount of clear mucoid discharge seen on the floor of both nostrils. In

Volunteer III.—Age 26 years. Bronchitis (?) 1928. Tonsilitis January, 1930. One cold during the past 2 years. Physical and nose and throat examinations normal except for slight irregularities in the nasal septum. Five-day control period during which an herpetic vesicle appeared on the lip and then retrogressed. Inoculated on 6/12/30 with a Berkefeld V filtrate of nasopharyngeal washings from Subject B. No symptoms of signs of an upper respiratory infection developed; on 6/17/30 was discharged. Aerobic cultures from the nasopharynx negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. The anaerobic culture showed no growth. White blood cell counts and the urine showed no abnormalities.

Volunteer IV.—Age 20 years. Pneumonia in 1916. Six colds during the past two years. Physical examination normal. Nose and throat examination showed questionable chronic tonsillitis. Five-day control period. On 6/12/30 inoculated with the Seitz filtrate of nasopharyngeal washings from Patient B. 36 hours later complained of sense of dryness, fullness and irritation in throat. Nose and throat at this time showed no changes and by the next day the symptoms had disappeared. Discharged on 6/17/30. Atypical hemolytic streptococci, type Beta, isolated in every culture from the nasopharynx. Non-hemolytic *H. influenzae* in the 5th day of the control period and on the 3rd day after inoculation. All anaerobic cultures remained sterile. White blood cell counts and the urine remained normal throughout.

Volunteer V.—Age 24 years. Two colds during the past 2 years. Physical and nose and throat examinations normal. Three-day control period. Inoculated on 6/17/30 with Berkefeld V filtrate of nasopharyngeal washings from Patient D. 54 hours after this, dryness, fullness and irritation in throat. Examination of nose and throat showed no significant changes. The symptoms rapidly disappeared; discharged on 7/21/30. The aerobic cultures from the nasopharynx negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. Anaerobic cultures showed Group IV organisms both before and after inoculation. The white blood cell counts and the urine were normal throughout.

Volunteer VI.—Age 21 years. Tonsillitis 1927. Acute frontal sinusitis 1927. Two colds during the past 2 years. Physical examination normal. Nose and throat examination showed a deflected septum and a mild chronic infection in the right tonsil. Three-day control period. The subject was inoculated on 6/17/30 with the Seitz filtrate of nasopharyngeal washings from Patient D. On 6/19/30, 50 hours after she complained of a sensation of dryness, fullness and of irritation in throat; nasal passages were obstructed. Slept with difficulty. 6/20/30—Developed a slight watery nasal discharge. Towards evening this became more marked and a high-grade of nasal obstruction was present. 6/21/30—Marked nasal obstruction and a moderate amount of mucoid nasal discharge, with slight headache and malaise. Temperature 99.2°F. (by mouth). Examination showed moderate hyperemia and swelling of nasal mucous membranes and a moderate

was normal. The glands at the angles of the jaw were tender. Temperature rose to 99.6°F. (by mouth). 6/29/30—The nasal discharge was mucopurulent and nasal obstruction less marked. No malaise. A slight conjunctivitis; eyes tired easily. The glands in the neck still tender. Forty gauze handkerchiefs were used during the day. 6/30/30—The nasal discharge much thicker and decreased in amount. Nasal obstruction intermittent. Crusting had commenced. No malaise. 7/1/30—Moderate anterior and posterior nasal discharge. Slight obstruction, and more marked crusting. Nose and throat examination: The remains of a rapidly clearing acute nasopharyngeal process. At the patient's request, discharged on this day. Non-hemolytic *H. influenzae* isolated in cultures from nasopharynx on the 3rd day of the control period and upon the 4th day after inoculation. *D. pneumoniae*, hemolytic *H. influenzae* and hemolytic streptococci, type Beta, not isolated. Anaerobic cultures showed Group IV and XIV organisms during the control period and Group IV and III A after inoculation. Cultures of the Seitz filtrates negative. The white blood cell counts and the urine were normal throughout.

Volunteer XI.—Age 20 years. Tonsillectomy and adenoidectomy in 1916. Two colds during the past 2 years. Physical examination was normal except for a small colloid goiter and a marked respiratory arrhythmia. Nose and throat examination showed both tonsil and adenoid remnants. Two-day control period. Inoculated on 6/28/30 and again on 6/29/30 with Seitz filtrates of nasopharyngeal washings from Volunteer X. 70 hours after this, marked lassitude, headache and backache; remained in bed. Temperature 100°F. (by mouth). Nose and throat examination showed poor breathing space on the left. The mucous membranes of the left lower inferior turbinate injected and swollen and a small amount of mucoid discharge on this side. 7/2/30—Moderate nasal discharge and obstruction and slight sore throat. Nose and throat examination showed swelling and injection of the mucous membranes in the nasal passages and a small amount of thick mucoid discharge. The pharynx was injected. Two hemorrhagic spots seen on the buccal mucous membrane of right cheek. 7/3/30—The nasal symptoms and signs remained unchanged. During the day suffered from generalized malaise, headache, backache, and a sharp attack of diarrhoea, accompanied by some abdominal discomfort. 7/24/30—The nasal findings were unchanged. Marked lassitude and moderate backache. No diarrhoea. 7/5/30—Moderate malaise and moderate diarrhoea during the morning. Temperature 100.4°F. (rectal). Much less nasal discharge and nasal obstruction present. Examination of the nose and throat showed that the rhinitis was clearing. Discharged on 7/6/30. The urine was normal throughout. No abnormal bacteriological findings were noted in the stool examination made during the periods of diarrhoea. The aerobic cultures from the nasopharynx were negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. Anaerobic culture showed Group IV and XVII organisms during the control period and Group IV organisms after inoculation. Cultures of the Seitz filtrates were negative.

the nasopharynx the adenoid tags were injected and swollen. Posterior wall of the pharynx completely covered with clear mucoïd discharge. 6/27/30—Nasal discharge more purulent in character and nasal obstruction more marked. Constitutional symptoms had disappeared but hoarseness had appeared. Nose and throat examination was unchanged. 6/28/30—Nasal discharge greatly lessened in amount and quite thick; nasal obstruction intermittent. A moderate productive cough with small amounts of tenacious mucoïd sputum developed and hoarseness was more marked. Examination: Nose—no discharge or swelling seen. The mucous membrane of the nasopharynx was injected. The pharynx was moderately injected with some mucoïd discharge over the entire posterior wall. The mucous membrane of the larynx slightly injected. Volunteer was forced to leave on this date but from subsequent reports was completely well within 5 days. Aerobic cultures from nasopharynx negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. Anaerobic cultures showed Group IV organisms both before and after inoculation. The white blood cell counts and the urine were normal throughout.

Volunteer IX.—Age 22 years. Influenza in 1918. Tonsillectomy and adenoidectomy in 1923. Three colds during the past 2 years. Physical examination showing nothing abnormal. Nose and throat examination was essentially normal. Three-day control period. Inoculated on 6/24/30 with a Seitz filtrate of nasopharyngeal washings from Patient E, on 6/25/30 with a mixed Seitz filtrate from Patients F and G and on 6/26/30 with a Seitz filtrate from Patient H. No definite symptoms or signs resulted from these inoculations; discharged on 7/1/30. Non-hemolytic *H. influenzae* isolated in cultures from nasopharynx on 1st day of control period and on 2nd, 3rd and 4th days after inoculation. *D. pneumoniae*, *Streptococcus haemolyticus*, type Beta, and hemolytic *H. influenzae* not isolated at any time. Anaerobic cultures showed Group IV organisms both before and after inoculation. The white blood cell counts and the urine were normal throughout.

Volunteer X.—Age 20 years. Nasal polyps removed in 1916. Tonsillectomy and adenoidectomy in 1929. Two colds during the past 2 years. Physical examination normal except for marked deafness in left ear. Nose and throat examination normal. Three-day control period. Inoculated on 6/24/30 with a Berkefeld V filtrate of nasopharyngeal washings of Patient E, on 6/25/30 with a mixed Berkefeld V filtrate from Patients F and G, and on 6/26/30 with a Berkefeld V filtrate from Patient H. 43 hours after the first inoculation, nasal obstruction, watery nasal discharge, sneezing and a slight feeling of malaise. Did not sleep very well because of nasal obstruction and on 6/28/30 had a profuse bilateral nasal discharge, nasal obstruction, hoarseness, a sore throat, sneezing and tender glands at angle of jaw on left side. During the day used seventy-four gauze handkerchiefs. Examination showed that the left inferior turbinate was greatly swollen causing marked obstruction on the left side. The mucous membranes of the nasal passages were injected and sticky mucoïd nasal discharge present in both nostrils. The pharynx

Volunteer XIV.—Age 18 years. Influenza in 1928. Tonsillectomy and adenoidectomy in 1921. Two colds during the past 2 years. Physical examination showed nothing abnormal. Nose and throat examination showed a deflection of the septum and slight hyperemia of the inferior turbinates; one hemorrhagic spot on left buccal mucous membrane. Five-day control period. Inoculated twice on 7/14/30 and once on 7/15/30 with Seitz filtrates of nasopharyngeal washings from Patient I. 24 hours after the first inoculation, hoarseness, slight soreness in the region of the uvula, a thin watery nasal discharge and nasal obstruction. 7/16/30—Signs and symptoms were increased in intensity. Examination: The right inferior turbinate was enlarged and congested, the right middle turbinate pale. The mucous membranes in the left nostril hyperemic. A small amount of mucoid discharge present. The throat was moderately injected. The glands at the angles of the jaw were slightly enlarged and tender. 7/17/30—During the day abundant anterior and posterior nasal discharge and constant nasal obstruction. Examination: The mucous membranes of the nasal passages were hyperemic, and the right turbinate enlarged. More discharge than on the previous day. The pharynx was hyperemic. 7/18/30—Severe frontal headache. Temperature was 100.8°F. (rectal). Nasal symptoms and signs remained unchanged. 7/19/30—No headache. No nasal discharge and obstruction had decreased. Temperature 100°F. (rectal). Examinations revealed no changes suggestive of sinus involvement. 7/20/30—Practically no nasal discharge. 7/21/30—No nasal discharge. Nose and throat examination was similar to that of entry. Hemolytic streptococci, type Beta, were isolated in all cultures from the nasopharynx. Aerobic cultures from the nasopharynx were negative for *H. influenzae* and *D. pneumoniae* throughout the experiment. Anaerobic cultures showed Group IV organisms both before and after inoculation. Cultures of the Seitz filtrates negative. A slight rise in the white blood cells upon the 3rd, 4th and 5th days after inoculation. The urine was normal throughout.

Volunteer XV.—Age 21 years. Pneumonia in infancy. Tonsillectomy in 1913. Influenza in 1918. Three colds during the past 2 years. Physical and nose and throat examinations normal. Inoculated twice on 7/16/30 and once on 7/17/30 with Seitz filtrates of nasopharyngeal washings from Volunteer XIV. On 7/18/30 4 hours after the first inoculation, a sensation of dryness and irritation in throat, thin watery nasal discharge, nasal obstruction, frontal headache and slight malaise. Twenty-five gauze handkerchiefs were used during the day. Examination revealed no changes in the nose. The lymphoid follicles on the posterior pharyngeal wall were larger and redder than previously. Two hemorrhagic spots on the left buccal mucous membranes. A small tender gland at the angle of the jaw on the left side. 7/19/30—The nasal discharge profuse and nasal obstruction more marked. Throat sore, hoarseness and a slight cough, with loss of taste. Fifty gauze handkerchiefs were used during the day. Examination showed definite congestion of mucous membranes in the nasal passages. A large amount of thin mucopurulent discharge in the left nostril. The lymphoid follicles on the posterior pharyngeal

6/27/30	W.B.C.—	7120
6/28/30	"	7200 Inoculated with Seitz filtrate
6/29/30	"	6080 R.B.C. 4,320,000. Hgbn. 100 per cent
6/30/30	"	5360
7/ 1/30	W.B.C.—	5280 Nasal signs
7/ 2/30	"	6320
7/ 3/30	"	4560 Diarrhoea
7/ 4/30	"	4200
7/ 5/30	"	5320 Diarrhoea
7/ 6/30	"	6200

Volunteer XII.—Age 20 years. Two colds during the past 2 years. Physical examination showed nothing abnormal. Nose and throat examination showed a deflected septum and some enlargement of the posterior half of the right inferior turbinate. Four-day control period. Inoculated on 7/2/30 with a Seitz filtrate of nasopharyngeal washings from Volunteer XI. 41 hours after this was very tired, and nasal obstruction with a slight watery nasal discharge developed. Towards evening, became moderately hoarse. 7/5/30—A moderate nasal discharge and nasal obstruction. No constitutional symptoms with the exception of a temperature of 100°F. (rectal). Examination showed swelling and hyperemia of both inferior turbinates together with a small amount of mucoid discharge on the left. The vessels of the soft palate and pharynx were engorged. Two hemorrhagic spots present in the buccal mucous membrane on the left side. 7/6/30—Nasal discharge thicker, the nasal obstruction less marked, and crusting had begun. The nasal passages felt very raw, and examination showed increased injection of the nasal mucous membrane but less swelling. A small amount of thick, sticky discharge seen on the floor of the nostrils. The entire pharynx slightly hyperemic. 7/7/30—The nasal discharge had markedly decreased as had also the nasal obstruction. Marked crusting. 7/8/30—Practically no nasal discharge or obstruction. Examination revealed normal nasal passages. The pharynx was moderately hyperemic. Volunteer was discharged on this day. The aerobic cultures from the nasopharynx were negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. Anaerobic cultures showed Groups XVII and IV during the control period and Groups XVII and III A after inoculation. The white blood cell counts and the urine were normal throughout.

Volunteer XIII.—Age 28 years. Influenza in 1918. One cold during the past 2 years. Physical and nose and throat examinations normal except for two hemorrhagic spots on the buccal mucous membrane of the left cheek. Five-day control period. Inoculated twice on 7/14/30 and once on 7/15/30 with Seitz filtrates of the nasal washings from Patient I. No symptoms or signs of an upper respiratory infection developed and on 7/17/30 the volunteer was discharged. The aerobic cultures from the nasopharynx negative for hemolytic streptococci, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. A diphtheroid was isolated from the anaerobic cultures both before and after inoculation. The white blood cell counts and the urine were normal throughout.

pharynx hyperemic, the glands at the angles of the jaw enlarged and tender. 7/22/30—The lacrimation, headache and sore throat had disappeared. The acuity of hearing was diminished. The nasal discharge less abundant and more mucoid. Examination: There was less swelling and hyperemia of the mucous membranes in the nose. A small amount of thick, sticky nasal discharge present on both sides. The throat only mildly injected. The glands at the angles of the jaw just barely palpable. 7/23/30—The nasal secretion was decreased in amount and was mucopurulent in type. Nasal obstruction intermittent. Examination showed better breathing space on both sides. Less congestion of the nasal mucous membranes. A small amount of sticky discharge. The throat was slightly injected. 7/24/30—Very slight nasal discharge; intermittent, with crusting. Examination normal except for a slight swelling of the inferior turbinates. Discharged. Atypical *D. pneumoniae*, Type II, present in cultures from the nasopharynx on the 5th day of the control period and on the 1st, 2nd and 3rd days after inoculation. Non-hemolytic *H. influenzae* present in two cultures during the control period. The aerobic cultures from the nasopharynx negative for hemolytic streptococci, type Beta. Anaerobic cultures showed Groups VII and IV organisms both before and after the inoculation. Cultures of the Berkefeld W filtrates were sterile. The white blood cell counts and urine normal throughout.

Volunteer II (Reentry).—Age 22 years. This subject again selected as a volunteer after an attempted inoculation 5 weeks previous to this entry. During the interim she had been free from upper respiratory disease. Five-day control period. Inoculated twice on 7/19/30 and once on 7/20/30 with Seitz filtrates of the nasopharyngeal washings from Volunteer XV. No symptoms or signs of a respiratory infection developed although in addition to the inoculation she was exposed to Volunteer XVII during the entire period. Non-hemolytic *H. influenzae* isolated in cultures from nasopharynx on 2nd, 3rd, 4th and 5th days of the control period and on the 3rd, 4th, and 5th days after inoculation. The aerobic cultures were negative for *Streptococcus haemolyticus*, type Beta, and *D. pneumoniae*. Anaerobic cultures showed Group IV organisms both before and after inoculation. The urine was normal throughout.

Volunteer XVIII.—Age 22 years. Tonsillectomy and adenoidectomy in 1913. Adenoidectomy in 1917. Influenza in 1918. Two colds during the past 2 years. Physical and nose and throat examinations normal. Two-day incubation period. Inoculated twice on 7/21/30 with Berkefeld W filtrates of the nasal washings from Volunteer XVII. 27 hours after the first inoculation, a sensation of dryness, fullness and of irritation in throat, a slight watery nasal discharge, partial nasal obstruction, sneezing, hoarseness and a frontal headache. 7/23/30—The nasal discharge more profuse and nasal obstruction more marked, with sore throat, frontal headache, aching in the neck and drowsiness. Examination showed that the nasal mucous membranes were moderately congested. A small amount of mucoid nasal discharge on both sides. The glands at the angles of the jaw enlarged, and slightly tender on the right. 7/24/30—Nasal discharge thick,

wall slightly more enlarged and inflamed. 7/20/30—The nasal discharge was thick and was decreased in amount. Nasal obstruction intermittent. Hoarseness had about disappeared and coughing was infrequent. 7/22/30—Very little nasal discharge; practically no obstruction. Cough much improved. 7/23/30—No clinical remains of the infection. The nose and throat examination practically normal. *D. pneumoniae*, Type IV, in all aerobic cultures from the nasopharynx and non-hemolytic *H. influenzae* on the 4th day of the control period and upon the 2nd, 5th and 6th days after inoculation. No hemolytic streptococci, type Beta. Anaerobic cultures negative during the control period but showed Group III A organisms after inoculation. Cultures of the Seitz filtrates negative. White blood cell count slightly elevated on the 2nd, 3rd, 4th and 5th days after inoculation. The urine was normal throughout.

Volunteer XVI.—Age 20 years. Influenza in 1918. Tonsillectomy and adenoidectomy in 1920. Two colds during the past 2 years. Physical and nose and throat examinations normal. Five-day control period. Inoculated twice on 7/16/30 and once on 7/17/30 with Seitz filtrates of nasopharyngeal washings from Volunteer XIV. During the evening of 7/17/30 temperature was 100.8°F. (rectal), but there were no symptoms or signs of upper respiratory disease. 7/18/30—At noon, complained of nausea, abdominal discomfort, headache, backache, aching in muscles and was extremely listless; towards evening diarrhoea developed. 7/19/30—Diarrhoea was checked but the marked listlessness, headache, and backache remained. Examination of the nose and throat normal. 7/20/30—No diarrhoea. Patient still very listless. Temperature 100°F. (rectal). 7/21/30—Felt very well. 7/23/30—Volunteer was discharged. The urine was normal throughout. The aerobic cultures from the nasopharynx were negative for *Streptococcus haemolyticus*, type Beta, *H. influenzae* and *D. pneumoniae* throughout the experiment. Anaerobic cultures were negative both before and after inoculation. White blood cell counts were within normal limits.

Volunteer XVII.—Age 18 years. Tonsillectomy and adenoidectomy in 1920. Grippe in 1927 and 1929. Two colds in the past 2 years. Said that all of her colds were "grippy" in nature. Physical and nose and throat examinations normal except for one hemorrhagic spot on each cheek. Five-day control period. Inoculated twice on 7/19/30 and once on 7/20/30 with Seitz filtrates of the nasopharyngeal washings from Volunteer XV. 20 hours after the first inoculation, chilliness, weakness, irritability, aching in the eyes and neck, lacrimation, sneezing, a thin watery nasal discharge, partial nasal obstruction, loss of appetite and taste and soreness and rawness in the nasopharynx and nasal passages. Slept very poorly. 7/21/30—Patient looked ill, and complained of a frontal headache, photophobia, aching in the neck, sore throat, tender glands, profuse nasal discharge and nasal obstruction. Temperature 100.6°F. This individual used 174 gauze handkerchiefs during the first 24 hours of her cold. Examination showed that the mucous membrane of the nasal passages was hyperemic and on the right side marked swelling was present. A large amount of clear watery nasal discharge was seen. The

on this basis. In evaluating the results, the strictness of quarantine and the selection of a season in the year when "colds" are not prevalent, are two considerations of great importance. We believe that by selecting intelligent volunteers who cooperated in observing strict isolation we have been able to fulfill the first of these requirements. The tests were made in Baltimore, Maryland, during a period (June and July, 1930) in which the attack rate of all upper respiratory infections was between one and two per hundred persons per week, a particularly low incidence.*

The nine subjects ill with natural colds, from whom the inoculum was obtained, were selected with care. Yeast infusion broth was used for washing the nasopharyngeal passages of the experimental subjects because Grinnell (18) has demonstrated that it facilitates the passage of certain viruses through filters. The results of our experiments agree with his findings. In the earlier experiments we were able to confirm the findings of previous investigators with regard to the transmission of upper respiratory infection with Berkefeld V filtrates. In each instance these filtrates contained Gram-negative filter-passing anaerobic organisms.

Seitz filters of the Uhlenhuth model and Berkefeld W filters were used in obtaining bacteria-free filtrates. Mudd (17) has demonstrated that the Uhlenhuth model of the Seitz filter does not permit the passage of test bacteria or certain dyes. In our experience neither filter has been permeable to the minute Gram-negative filter-passing organisms present in nasopharyngeal washings, and in all instances the filtrates from the Seitz and Berkefeld W filters remained sterile when cultivated in the test media. This is not due to an inhibitory or bactericidal effect of the filter materials for it is possible to cultivate Gram-negative anaerobes from a Berkefeld V filtrate in a medium to which large pieces of the Seitz filter discs have been added. Filtration was accomplished under negative pressure by use of the laboratory vacuum and not more than 30 minutes elapsed between obtaining the washings and completing the inoculations. The time element is of importance as the incitant may perish rapidly when removed from its natural environment.

* We were able to utilize data from a study of upper respiratory infection in a group of one hundred families through the kindness of Dr. V. A. Van Volkenburgh.

nasal obstruction intermittent. Sore throat persisted. The appearance of the nose and throat had not changed. 7/25/30—The nasal discharge was lessened in amount. Crusting was present. Nasal obstruction was intermittent. Examination showed less congestion and discharge. The posterior pharyngeal wall was hyperemic. 7/26/30—Very little nasal discharge. Nasal obstruction intermittent. 7/27/30—Very slight amount of nasal discharge, and practically no obstruction. 7/28/30—Discharged. Nose and throat examination was essentially normal. *D. pneumoniae*, Type IV, were isolated in cultures from the nasopharynx throughout the experiment. No *Streptococcus haemolyticus*, type Beta, or *H. influenzae* isolated in the cultures from the nasopharynx. Anaerobic cultures during the control period negative, but after inoculation Groups IV and VII organisms were isolated. White blood cell counts and urine normal throughout.

Volunteer XIX.—Age 23 years. Tonsillectomy and adenoidectomy in 1914. Influenza in 1924. Two colds during the past 2 years. The nose and throat examination essentially normal except for remnants of tonsil tissue on the posterior pharyngeal wall. Two-day control period. Inoculated twice 7/21/30 with Berkeley W filtrates of nasal washings from Volunteer XVII. 24 hours later, sore throat and headache; very listless. Temperature 100°F. (rectal). 7/23/30—Had not slept well. A thin watery nasal discharge present, with intermittent nasal obstruction. Sore throat persisted. Temperature 100°F. (rectal). Examination showed no changes. 7/24/30—Sore throat remained unchanged. Backache was present. The nasal discharge more profuse and nasal obstruction more prominent. The subject was hoarse and complained of substernal pain; remained in bed the entire day. Temperature 100.4°F. (rectal). Examination showed hyperemia and swelling of the nasal mucous membrane. A small amount of mucoid nasal discharge. Ten hemorrhagic spots scattered over the uvula. The glands at the angles of the jaw enlarged and tender. 7/25/30—Felt much better. The nasal discharge had decreased and the nasal obstruction was intermittent. Temperature 100.1°F. (rectal). The nose and throat examination was unchanged. 7/28/30—Discharged. The nose and throat examination was normal. Non-hemolytic *H. influenzae* isolated in cultures from the nasopharynx on the 1st, 2nd, 3rd, 4th and 6th days after inoculation. Aerobic cultures of the nasopharynx negative for hemolytic streptococci, type Beta, and for *D. pneumoniae*. Anaerobic cultures were negative both before and after inoculation. White blood cell counts and urine normal during the entire period.

DISCUSSION

This study has to do with the transmission of upper respiratory infections (common colds) to human volunteers. Judging from their past histories, the individuals selected as subjects for the test varied moderately in respect to the number of natural "colds" which they had within the past 2 years, but no attempt was made to select them

In all, eleven out of twenty inoculations resulted in upper respiratory infection. During the 7 week period in which the inoculations were done the prevalence of upper respiratory infection in a group of one hundred families under constant and close observation was at the rate of 2.13 per cent per week. With this rate of prevalence we might expect to have had about 5 per cent of our subjects develop natural "colds" within the period of observation which in each individual was about 10 days, if they had been at large in their normal surroundings. However, this rate of prevalence would naturally be greatly decreased by the strict isolation of the volunteers so that it would be difficult to determine the expected rate of prevalence. This is to be compared with the actual occurrence of eleven colds in nineteen people, and moreover, all colds occurred within 3 days of inoculation with no instance of a cold preceding inoculation or following it after an interval of 3 days.

In general the upper respiratory affections developed by the volunteers subsequent to inoculation were attended at their inception by mild constitutional disturbances. In six of the successful transmissions there was a slight fever during the initial stages of the infection. In three no febrile reaction was observed. The remaining two developed slight fever late in the course of the disturbance. The incubation period (as measured from the time of the first inoculation to the onset of symptoms) varied from 20 to 70 hours.

The earliest symptoms consisted of sensations of fullness, dryness and irritation in the nasopharynx. Some subjects complained of an actual sore throat. Pharyngitis was generally present and appeared to be associated with the spread of the virus as no changes in the aerobic flora could be demonstrated in the nasopharynx. In the early stages of the infection, the nasal discharge was thin and watery but it varied greatly in amount. The objective changes in the nose consisted of hyperemia, swelling of the mucous membrane and nasal discharge. These signs were found to change rapidly within short periods of time. The frequent nose and throat examination aided us in diagnosing the affection, since definite evidence of nasopharyngeal abnormality was required before an experiment could be considered positive. None of the volunteers developed complications secondary to their upper respiratory infections. This, we believe, can be attrib-

The studies of the aerobic bacterial flora in the nasopharynx showed no striking differences between the control period and the period of the upper respiratory infection. Certain of the volunteers did not harbor any of the organisms under observation; others were consistent carriers throughout the period of investigation. It is concluded that the particular aerobic bacteria studied played no part in the production of the upper respiratory infection.

Anaerobic cultivation of the Berkefeld V filtrates of the nasopharyngeal washings from the subjects ill with natural colds yielded positive results in all instances. No constant predominating organism was found.

Nineteen individuals acted as subjects and twenty inoculation tests were performed. In the first ten the volunteers were divided into pairs, one of each pair receiving Berkefeld V filtrates and the other Seitz filtrates of the nasopharyngeal washings from individuals ill with natural "colds." Two of the subjects receiving Seitz filtrates and two receiving Berkefeld V filtrates developed "colds." These tests confirmed the observation of earlier observers in regard to the transmission of "colds" by means of Berkefeld V filtrates and also demonstrated that the infection can be transmitted by bacteria-free filtrates.

In the second group of ten tests serial transmission of the infection through two and four passages was accomplished with bacteria-free filtrates. In the first instance the source of infectious material was Volunteer X whose infection developed subsequent to inoculations with Berkefeld V filtrates from the nasopharyngeal washings of an individual ill with a natural "cold." The affection was transmitted in serial passage from Volunteer X through Volunteers XI and XII by means of Seitz filtrates. The source of material for the second serial transmission test was Patient I and the transfer of her "cold" to Volunteer XIV and the subsequent serial passage of this infection through Volunteers XV and XVII was accomplished with Seitz filtrates. A fourth generation passage of the affection from Volunteer XVII to Volunteers XVIII and XIX was made with Berkefeld W filtrates. Thus two single transfers and serial transmission through two and four generations have been made with bacteria-free filtrates and the earlier observation in regard to transmission of "colds" with Berkefeld V filtrates confirmed.

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uted to the time of the year in which the experiments were conducted, and to the fact that the subjects were protected by quarantine against potentially pathogenic microorganisms. A close check was kept upon each volunteer after leaving the hospital and in no instance did an acute exacerbation of the infection occur. There were no abnormal findings in the urines of the subjects during the experimental period and the records of the white blood cell determinations do not show any significant changes attributable to the presence of infection.

Following the inoculations two of the volunteers developed short, but nevertheless distinct attacks of diarrhoea accompanied by malaise and prostration. One was suffering from an experimental upper respiratory infection and the other did not show any signs of nasopharyngeal involvement. In one case a definite leukopenia developed and in the other the white blood cell count was decreased but did not reach a leukopenic stage. Stool cultures from both individuals showed a normal flora. At the time there were no other individuals ill with a similar disease in the hospital. The clinical picture presented by these two volunteers closely resembles the influenzal syndromes which are frequently observed during waves of upper respiratory infection. We present these two interesting cases but we do not feel that any definite conclusions can be drawn from them at this time.

CONCLUSION

Experimental upper respiratory infections similar to "common colds" were transmitted singly and in series through two and four passages in nine out of fifteen persons, by intranasal inoculations with bacteria-free filtrates of nasopharyngeal washings obtained from individuals ill with natural "colds." These observations conform with those reported by previous workers and lend further support to the view that the incitant of the "common cold" is a filtrable virus.

We wish to express our sincere appreciation of the kind cooperation of Dr. Winford H. Smith during this study.

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which are non-hemolytic and which require for growth both of the accessory factors X and V.

The difficulty of identifying and classifying strains of this group is further increased by the fact that many individual strains, when grown under artificial conditions, may show temporary or permanent variations in certain characters. The colony which is generally considered typical for *Hemophilus influenzae* is small, round, discrete, translucent, and finely granular on the surface. Certain writers have, however, described colonies which differ in appearance from those described as typical, and in a few instances the writers have found this atypical colony formation associated with variations in morphology of the individual bacteria and with certain modifications of other biological characters.

A number of writers have stated that the colonies formed by strains derived from cases of meningitis, septicemia, or arthritis are more opaque than those described as typical, (Cohen (9), Henry (10), Taylor (11), Grekowitz (12), and others). Scott (4) has stated that the colonies formed by certain strains derived from the healthy nasopharynx, or from cases of meningitis or arthritis are not only slightly more opaque than the typical colonies but that they "are often distinguishable by a bluish iridescence in oblique light." He stated that the bacilli of these strains are of "abnormal size." He also noticed that these properties were not fixed but, that after growth on artificial media, these strains "may be indistinguishable from some, at least, of the strains of respiratory origin."

Kristensen (13) has mentioned the formation of "coarse" colonies and he states that the coarse appearance of the colonies is undoubtedly associated with a coarse morphology of the individual bacteria, particularly with the occurrence of thread forms.

Certain writers have noted other unusual properties of strains from pathological sources. For instance, Wollstein (14) has observed that the bacteria of strains from the cerebrospinal fluid of cases of meningitis autolyze more readily than the bacteria of strains from the respiratory tract. A number of writers (15, 16, 17, 18, 19, 12) have mentioned that influenza bacilli from pathological sources are pleomorphic. Wollstein (20), however, noted that in young cultures of freshly isolated pathological strains most of the bacteria are short and uniform rods. If growth continues in these cultures for some time the uniformity becomes less evident, and after these strains have been subcultured for a long time even the organisms of the young cultures are pleomorphic (14). Cohen, Ritchie (21), and Henry have also observed that in young cultures of meningitis strains the bacteria appear as uniform rods while in older cultures they are pleomorphic.

Cohen, Henry, Ritchie, Parker and Parker (22), Nabarro and Stallman (18), Taylor, and Wollstein have observed that strains isolated from pathologic sources are usually more pathogenic for animals than are the usual respiratory strains, and some investigators (22, 23, 24) have observed that during subculture these strains lose their pathogenicity. Wollstein, Povitsky and Denny (25), Rivers and Kohn (26) have found a considerable degree of immunological relationship between the strains isolated from cases of meningitis.

BOSTON PSYCHOPATHIC HOSPITAL

VARIATION AND TYPE SPECIFICITY IN THE BACTERIAL SPECIES *HEMOPHILUS INFLUENZAE*

By MARGARET PITTMAN, Ph.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

PLATE 19

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During the course of a study of Pfeiffer bacilli, strains were grown on special transparent agar and it was observed that when one particular strain was grown in this way two kinds of colonies developed. The colonies of one kind were like those usually described as typical for this organism while the colonies of the other kind were opaque and were very iridescent when viewed by oblique transmitted light. It was also observed that the organisms forming the colonies of one kind differed in morphology from the organisms forming colonies of the other kind (1). Further study indicated that the phenomenon was undoubtedly an example of variation or bacterial dissociation. During recent years much new and important knowledge concerning variations in bacteria of other species has been obtained (2) and it has seemed important to study in greater detail, in the light of this new knowledge, the variations occurring among the so-called influenza bacilli, or Pfeiffer bacilli.

The bacteria of this group do not form a well characterized bacterial species, and it has long been recognized that individual strains differ from one another in morphology and virulence, in the appearance of the colonies which they form, in their ability to form indole, in power to ferment sugar and to induce hemolysis, in their immunological reactions, and even in their requirements for growth in artificial cultures. The literature relating to the biological characteristics of the bacteria forming this heterogenous group has been recently reviewed by Jordan (3) and Scott (4). In 1920 the Committee on Nomenclature of the American Association of Bacteriologists (5) proposed the name *Hemophilus influenzae* for this group of organisms. Soon after this, more accurate studies of the growth requirements of the organisms of this group were made (6, 7, 8). In the present paper the term *Hemophilus influenzae* is employed to include only those organisms

When cultures of these 15 strains were grown on plates prepared with this medium, the colonies were large, sometimes 3 mm. in diameter. The surfaces of the colonies were smooth and mucoid and the edges were continuous. If the colonies were close together, they tended to coalesce. They were slightly opaque and in strong light, obliquely transmitted, they were markedly iridescent. This iridescence is quite distinct from the bluish luster sometimes exhibited by other colonies of *Hemophilus influenzae*.

It was frequently observed that as these strains were subcultured there appeared among these opaque colonies other colonies which resembled the "typical" *Hemophilus influenzae* colony. These new colonies which appeared among the iridescent colonies were usually smaller and more discrete; they were translucent and non-iridescent in transmitted light. The surface varied in roughness; some were only slightly granular with the margins slightly indented, others were deeply wrinkled with the contours broken by deep serrations. When cultures were made from these rough colonies, it was found that all the colonies which developed were granular like the mother colony. It was obvious, therefore, that these cultures were variants of the original strains from the patients. Some of the conditions under which this variation or dissociation takes place will be described later.

The phenomenon which has been observed in the case of these organisms so closely resembles the formation of variants of *Pneumococcus* (28) and other organisms that it has seemed justifiable to adopt a similar terminology and to speak of the 15 strains just described, which form smooth colonies, as S strains, and the strains derived from them forming granular colonies, as R strains. The smooth iridescent colony will hereafter be spoken of as an S colony, and the rough translucent colony will be called an R colony.

The differences in size and opacity between colonies of the two kinds are illustrated in Fig. 1. But the most distinguishing characteristic of the S colony, the iridescence, is not shown in the photograph. So marked are these differences that when colonies of different kinds coalesce the S colonies appear "moth-eaten" and the presence of a bacteriophage is suggested. It should be emphasized, however, that these differences only appear striking in the case of cultures grown on Levinthal transparent agar plates. On blood or chocolate agar the

In the present study, 155 strains of hemoglobinophilic bacilli were isolated from comparatively widely distributed sources. Only 97 of these strains, however, were non-hemolytic and required both X and V factors for growth. Of these 97 strains 82 have never been observed to form the atypical opaque colonies previously mentioned. Whether under suitable conditions these strains would also produce atypical colonies cannot be stated at present. Fifteen of the strains, however, at the time they were isolated, when grown on transparent agar plates produced opaque iridescent colonies. The sources¹ of these fifteen strains were as follows:

Seven strains were isolated in pure culture from the spinal fluids of meningitis patients. One of these patients suffered from arthritis preceding the onset of the meningitic symptoms. In this case influenza bacilli grew in cultures from the joint as well as from the blood, and these strains were identical with the strain from the meninges, identical not only in their serological reactions but also in biochemical reactions and cultural characteristics. Two strains were obtained in pure culture from the blood of cases of pneumonia. One strain was isolated in pure culture from the purulent exudate in a case of empyema associated with pneumonia. Two strains were isolated from the sputum of patients suffering from atypical pneumonia. In the sputum of both of these patients pneumococci of Group IV were also present; the influenza bacilli, however, predominated. Two strains were isolated from the throats of patients suffering from pharyngitis. In the cultures from the throats of both of these patients at least 90 per cent of the colonies were of influenza bacilli. In these cultures typical influenza bacilli colonies were present as well as opaque iridescent ones. One strain was isolated from the nose of a monkey suffering from tuberculosis.

For determining the appearance of colonies, all strains were grown on Levinthal's transparent medium (27).

In the preparation of this medium the authors' directions were slightly modified in order to avoid the need of sterilization by filtration. 4 per cent sterile nutrient agar was employed, and just before pouring plates, the agar was melted, partially cooled, and mixed with an equal volume of Levinthal's broth (prepared by boiling 10 per cent blood broth for 5 minutes, filtering through paper and then through a Berkefeld candle).

¹ Certain of these strains have been received from Dr. Martha Wollstein of the Babies Hospital, New York City; Dr. Anne G. Kuttner of the Pediatric Clinic of Johns Hopkins Hospital; Dr. J. D. Trask, Jr., of the Department of Pediatrics of Yale University, and Dr. D. W. Weiss of the Department of Pediatrics of Washington University, St. Louis, to whom the author is gratefully indebted.

Pathogenicity of S and R Strains

As is well known the bacteria of the *Hemophilus influenzae* group, when inoculated into animals, do not have a marked tendency to invade the blood and tissues. Nevertheless, the inoculations are frequently followed by marked toxic reactions, and death not infrequently results, even though at autopsy the cultures may be sterile. Individual animals also differ markedly in their susceptibility to the action of these organisms and their products, so that it is difficult to determine the minimal lethal dose with great accuracy. In comparing the pathogenicity of different strains, therefore, it is not sufficient to determine the least amounts of the cultures that will cause the death of animals, or even to compare the intervals of time elapsing between the injection of given amounts and the fatal outcome. Other circumstances are also significant. When the injections are made into the peritoneum it has been found to be important to determine whether, at the time of death, living organisms are still present, or whether they have disappeared; and, if they are still present, to observe the morphological changes which they have undergone; also to note the character of the cellular reactions in the exudate, and to establish whether or not invasion of the blood has occurred. In comparing the virulence of S and R strains, observations of all of these circumstances have been made, and the results of many experiments are given in a condensed form in Table I.

In all the experiments 20 hour broth cultures have been employed. By this method the dosage could be more accurately determined than by using suspensions of organisms grown on plates. The medium used was Levinthal's broth (prepared with 2½ per cent blood) and this was introduced into Erlenmeyer flasks in small amounts, so as to give an extensive surface exposure to the air. Rabbits, rats, and mice were used for the experiments. The injections were made intraperitoneally in the case of the mice and rats, and intravenously and intracutaneously in the rabbits.

When fatal doses of influenza bacilli are injected into animals, they usually appear very sick within a few minutes, the breathing becomes labored, they refuse to eat, and frequently diarrhea occurs. Within 2 hours the eyes become watery. In many cases especially in mice the conjunctival exudate later becomes purulent. Death usually occurs within 24 hours. At autopsy there is found hyperemia

presence of R colonies can only be detected with very great difficulty. Moreover, the differences between the colonies of the two kinds are striking only in young cultures. If growth has continued in the incubator over 24 hours, or if the plates have been allowed to remain at room temperature for several days, the S colonies become as translucent as the R colonies. Even on the old plates, however, the surface of the S colonies remains smooth, provided sufficient moisture is present to prevent drying. When touched with a platinum wire, the S colonies are soft and yielding, while the R colonies are firmer and more tenacious. The bacteria from the S colonies can be readily and uniformly suspended in salt solution, those from the R colonies are suspended in salt solution with greater difficulty and they tend to agglutinate spontaneously.

Morphology of the Bacteria of the S and R Strains

In stained preparations made from a young R colony, the bacteria are of various sizes and lengths, short rods and also long thread forms being seen. In preparations made from a young S colony, on the other hand, practically all of the bacteria appear as short rods, almost uniform in size. In older plate cultures, the bacteria from the S colonies also become pleomorphic and long threads and bizarre forms are seen. This tendency of the S bacteria to become pleomorphic is, however, more marked in broth cultures than in plate cultures. What seems to be of special significance is the fact that the S bacteria, when carefully stained and studied, are found to be surrounded by capsules which, though usually less thick than those of pneumococci, are, nevertheless, perfectly distinct and definite. Welch's (29) and Muir's (30) stains have been found most useful in demonstrating them. The morphology of S and R bacteria is shown in Figs. 2 to 5. That hemoglobinophilic bacteria from cases of meningitis are encapsulated was recently suggested by Grekowitz (12). He observed that the stained bacteria were surrounded with a halo. The bacteria of the S colonies also undergo autolysis more readily than do those of the R colonies. This may explain the irregular staining and the occurrence of shadow forms in old S cultures.

they are sterile. When stained preparations of the peritoneal exudate are made, it is found that when R strains have been injected the bacteria which are present frequently do not appear as typical influenza bacilli, but are swollen, at times appearing as almost round, globoid, irregularly staining masses (Fig. 7). These bodies resemble the swollen forms of *Vibrio cholerae* which Pfeiffer (31) described as occurring in the peritoneal exudate of immunized guinea pigs. Similar changes in the bacteria are sometimes found in the exudates of animals inoculated with S strains, but in this case the changes are not of so extreme a degree nor have so many of the bacteria undergone this modification in form (Fig. 6). The exudate in an animal inoculated with an R strain is usually scant and contains many leucocytes, while that from an animal inoculated with an S strain is more viscous and few leucocytes are present. There is also observed a difference in the persistence of the bacteria in the blood, when injections of strains of the two kinds are made intravenously. In the case of organisms of the R variety, few bacteria are present in the blood at autopsy, or they may have entirely disappeared.

It has also been found that when injections of the strains of the two kinds are made into the skin of rabbits, there are even more marked differences in the effects produced than when the inoculations are made intraperitoneally or intravenously. This method of study has the additional advantage in that several inoculations may be made at one time into a single animal, and the effects of different strains may thus be observed in the same animal. In this way the influence of individual differences in susceptibility of different animals may be eliminated.

In all the inoculations, 0.1 cc. of a broth culture was introduced as superficially as possible into the shaven skin. The course of the reaction following the inoculation of an S culture is as follows. The fluid is slowly absorbed and the involved area appears blanched for several hours, after which it becomes erythematous. Within 24 hours the affected area measures 2 to 3 cm. in diameter. In the center there is a small area of necrosis and the skin about this is very red and edematous. At the end of 48 hours the reaction begins to decrease in intensity, and the involved area of the skin becomes smaller so that in a week it measures about 1 cm. in diameter. The skin is now only slightly red, but there is definite induration and only after 10 to 20 days has the reaction entirely disappeared. On the other hand, the skin reaction which follows the injection of a culture of an R strain is less

and frequently hemorrhage in the tissues. These are especially marked in the case of rabbits, in the thymus, lungs, and mesentery. Microscopic examination of the lungs reveals the presence of edema and hemorrhage.

In comparing cultures of S and R strains, it has been found that in all the animals studied the minimal lethal dose of the S culture has been uniformly smaller than that of the corresponding R culture. Indeed

TABLE I
Results of Inoculations of S and R Strains

Animal	Strain	Route of inoculation	M.L.D.	Organisms in culture from		Stained preparations of peritoneal exudate		
				Peritoneum	Heart's blood	Bacteria	Globoïd forms	Leucocytes
	<i>culture</i>		<i>cc.</i>					
Mouse	S	Intraperitoneal	0.1-0.5	Many	Many	Many	Rare	Few
"	R	"	1.0-2.0 or avirulent	Few	Few or none	Few	Many	Many
Rat	S	"	0.25-1.0	Many	Many	Many	Rare	Rare
"	R	"	2.0-3.0 or avirulent	Few	Few or none	Few	Many	Many
Rabbit	S	Intravenous	0.5-1.0		Many			
"	R	"	2.0-3.0 or avirulent		Few or none			
	<i>filtrate</i>							
"	S	"	1.0-3.0		—			
"	R	"	3.0-5.0 or avirulent		—			

many R strains have been found to be completely avirulent. In animals dying after intraperitoneal injections, it has been found that, if the strains injected were of the S variety, organisms are still present in the exudate, while if R organisms were injected, few or no bacteria remain. A marked difference between the bacteria of the two kinds as relates to their invasion of the blood has also been noted. If the cultures injected were of the S variety, many organisms grow from the cultures of the blood at autopsy, while if R organisms were injected, the cultures from the blood at autopsy show a very scanty growth, or

of the same strain a specific precipitation occurred. The same reaction occurred when washings of a plate culture were added to the serum. The bacteria were washed in salt solution and removed from the fluid by centrifugalization. On the other hand, when the same culture filtrate or bacterial washing fluid was added to an immune serum produced by the injection of R organisms, even though derived from the same S strain, no precipitation occurred. Moreover, no precipitation occurred when filtered R broth cultures, or washings from plate cultures of R strains were added to S immune serum. These observations indicated that in the culture fluids of S strains there exists a soluble substance, which reacts with the homologous immune serum, and that this substance is easily removed from the S bacteria by washing. On the other hand, no such substance in the R strain could be demonstrated, nor did the serum produced by the injection of R strains contain any antibodies effective against this soluble substance.

It was now important to determine whether this soluble substance was specific only as regards the homologous strain, or whether, so far as this reaction is concerned, all S strains are immunologically identical, or whether one soluble substance is specific for a certain number of strains, which thus would form an immunological type. Several groups of rabbits were, therefore, immunized, each one against one particular S strain.

For the purposes of this particular experiment, three groups of rabbits were immunized, one against the Strain 35S, one against 41S, and one against 51S. The best immune sera were obtained by giving repeated intravenous injections of living organisms which, immediately before the inoculation, were scraped from plates and suspended in 20 per cent normal rabbit serum. After the course of immunization had been completed, sera obtained from the rabbits were tested against the soluble substances of each one of the S strains used in immunization.

In making the tests, washings from plate cultures were employed, as they have been found to be not only more active but also more convenient to prepare than are the culture filtrates. The bacteria from a 20 to 24 hour Levinthal agar plate culture were suspended in 3 cc. of physiological salt solution. The mixture was immediately centrifugated at high speed and the clear supernatant fluid was used for the tests. Various dilutions of the fluid from the cultures of each of the three S strains were made. 0.4 cc. of each dilution was placed in a precipitin tube. To each tube was then added an equal quantity of serum, diluted with saline in the

severe, the erythema is much less marked, and the duration of the reaction is much shorter.

It has been observed also that there is a close correlation between the severity of the skin reaction produced by a given culture and the minimal lethal dose of this strain for laboratory animals. Strains which form very rough colonies, and which are apparently without virulence, as measured by intraperitoneal inoculation, have been found to produce no skin reactions, or very slight ones which fade within 48 hours; while those strains which produce less rough colonies, and which are moderately virulent, induce more marked skin reactions. Dold (32), working with different strains of streptococci, has also noted a close correlation between the severity of the skin reaction produced and the virulence of the culture.

Immunological Reactions of S and R Strains

Many investigators have attempted to determine the immunological relationships between various strains of influenza bacilli. In most of these attempts the method of agglutination with univalent sera has been employed, though studies employing complement fixation have also been made. While certain observers have been able to demonstrate some immunological relationships between certain cultures, it has not been possible to develop any useful or accurate method of grouping these organisms, such as has been arrived at in the case of pneumococci and Friedländer's bacilli.

The observations which have just been reported, especially the fact that S and R forms of influenza bacilli occur, and that the one form is apparently a variant of the other, and furthermore, that the bacteria of the S form are possessed of a capsule, have suggested that the mechanism underlying the immunological relationships of influenza bacilli may not be essentially different from that upon which the immunological relationships of pneumococci depend, and that the difficulties previously encountered in grouping these organisms may depend, to some extent at least, on the facts that the method employed has chiefly been that of agglutination, and that S and R strains were used indiscriminately in the testing. This supposition was supported by the observation that when the serum of an animal immunized against one of the S strains was added to the filtrate of a fluid culture

which are of Type b, has been previously mentioned. It is of interest that all of the seven strains² from cases of meningitis are of Type b. There is also in the laboratory a specimen of immune serum prepared, before this work was undertaken, by the injection of a rabbit with a strain of *Hemophilus influenzae*, also isolated from a case of meningitis. The strain itself now produces only R colonies but the immune serum has been found to be active against Type b strains.

The immune sera which were prepared by the injection of the S strains, were now used for testing the agglutination reactions of these various S strains. When the tests were carried out at 37°C. for 2 hours and overnight on ice, the same type specificity as revealed by the precipitin tests became evident. The bacteria of 35S, Type a, were agglutinated only in 35S serum, not in that produced by the injection of Strain 51S, Type b, and *vice versa*. This type-specific agglutination at 37° has been confirmed by many other tests of S organisms. The agglutinating powers of the sera produced by the injections of R organisms, and the agglutinability of the R strains in the various sera, however, have not given results which are so easy to interpret. At 37°, S strains are not agglutinated by any of the R sera. So far as tested, however, all R strains are agglutinated by all R sera.

When, however, instead of carrying out the agglutination reactions at 37°, the tests were made at 47°C. for 4 hours and overnight on ice, somewhat different results were obtained. Under these conditions there was observed no specificity of the reactions of the S strains as regards type. The Type b strain, 51S, was now agglutinated in Type a serum and both 35S, Type a, and 51S, Type b, were equally well agglutinated in Type b serum. Moreover, the S strains of both types were now agglutinated by all the sera produced by the injection of R organisms. Also all R strains, at this temperature, are agglutinated in all the sera, whether R or S of Type a or Type b.

There is one difference, however, as regards the character of the agglutination reaction, between the agglutination of the S culture in the type-specific S sera and all the other agglutination reactions. In

² Since the completion of this paper two other strains from cases of meningitis have been received. Both strains are of Type b. A precipitin test was also made with the spinal fluid from one of the patients and a positive reaction was obtained in Type b serum.

proportion of 2 parts of serum to 3 parts of saline. The tubes were placed in the incubator for 1 hour at 37°C. and then left on ice overnight, and examined on the following morning.

The results of this experiment are shown in Table II. It is seen that precipitin reactions occurred in the mixtures of the soluble substances of 41S and 51S with 41S serum and also in the mixtures of these soluble substances with 51S serum. Moreover, all these reactions were of equal intensity. On the other hand, the soluble substance from Strain 35S reacted only with its homologous serum, no precipitation occurred when the 35S serum was mixed with the soluble substance of either of the other strains. It was evident then that Strains 41S

TABLE II

Precipitation of the Soluble Substance of S Strains in Anti-S Serum

Serum	Antigen ¹	Final dilution of supernatant fluid							Controls
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	
35S	35S	++++	++++	++++	++++	++++	++	+	—
"	41S	—	—	—	—	—	—	—	—
"	51S	—	—	—	—	—	—	—	—
41S	35S	—	—	—	—	—	—	—	—
"	41S	++++	++++	++++	++++	++++	++++	+	—
"	51S	++++	++++	++++	++++	++++	++++	+	—
51S	35S	—	—	—	—	—	—	—	—
"	41S	++++	++++	++++	++++	++++	++++	+	—
"	51S	++++	++++	++++	++++	++	++	+	—

and 51S are of one immunological type, so far as could be tested by this method, while 35S is of an entirely different type. Other similar experiments have been carried out to determine the immunological specificity of each one of the 15 S strains so far isolated. It has been found that two of these strains are of the same type as 35S, and this type has been called Type a, while the 12 remaining strains are of the same immunological type as 51S, as tested by the precipitin reaction, and this type has been called Type b. It is of interest that of the three Type a strains, two are those which were isolated from cases of atypical pneumonia, and the third is one of those which were isolated from cases of pharyngitis. The source of the remaining strains, all of

Further studies, which are under way, it is hoped may throw more light on the question of the immunological relationships of this group of microorganisms.

Biochemical Reactions of S and R Strains

Extensive studies have been made to determine whether any differences exist between the S and R strains in relation to certain biochemical reactions. All of the strains, both S and R variants, have been found to produce indole and to reduce nitrates to nitrites.

The ability of the various strains to attack different sugars has been studied.

The sugars employed have been glucose, xylose, galactose, mannite, levulose, maltose, saccharose, lactose, and dextrin. The medium used has been Dunham's peptone solution to which were added the growth accessory factors for influenza bacilli, as in the preparation of Levinthal's broth. The various sugars were added to tubes of this medium, the tubes were inoculated with the strains to be studied, and were left in the incubator for 10 days.

The only sugars fermented by any of the strains were glucose, galactose, and xylose. All of the S strains fermented glucose. The twelve Type b strains also fermented galactose and all but one fermented xylose. On the other hand, only one of the Type a strains fermented galactose and none of them fermented xylose. The R strains fermented the same sugars as did the S strains from which they were derived. With none of the strains, however, was acid production very marked, and the fermentation reactions of a given strain were not entirely constant and regular, when the tests were repeated. This irregularity of fermentation reactions by strains of influenza bacilli has also been noted by others (Stillman and Bourn (35), Rivers and Kohn). While, therefore, there have been some differences noted in the fermentation reactions exhibited by strains of Types a and b, it is not likely that these are distinguishing characteristics of the two types of influenza bacilli.

Several observers have previously noted that influenza bacilli are soluble in bile or in solutions of bile salts (Sellards and Sturm (36), Neufeld and Etinger-Tulczynska (37)), though little attention has been paid to this observation in the literature dealing with this organism. Without knowledge of this phenomenon, it was noted that several S

the former case the agglutinated bacteria form a firm mass or disc at the bottom of the tube and this disc can not easily be broken up by shaking. In all the other agglutination reactions the bacteria form a loose precipitate which, on shaking, becomes easily separated into small granular masses which become distributed throughout the fluid.

In the light of the careful studies of the immunological relationships in another group of organisms, *Pneumococcus*, by Avery (33) and others, one may venture to offer a probable explanation of the phenomena just described. Influenza bacilli of the S variety undoubtedly produce a soluble substance which is specific for each type. The chemical nature of the soluble specific substance of Type a is now being studied by Dr. Goebel, and it has been found that this substance belongs among the carbohydrates, just as do the soluble specific substances of pneumococci and Friedländer's bacilli. The fact that influenza bacilli of the S form possess capsules, while those of the R form have no capsule and also form no soluble specific substance, suggests that the soluble specific substance is related to this morphological structure, as has been demonstrated to be the case with pneumococci and Friedländer's bacilli (34).

It has been shown that in the case of pneumococci the S forms possess not only a type-specific antigen, but also a species-specific antigen, the latter being the so-called nucleoprotein which forms a large part of the cell. Immunization with R pneumococci gives rise only to antibodies which react with all R pneumococci, no matter from which type they are derived. Immunization with S pneumococci gives rise mainly to the development of type-specific antibodies, but in all cases such sera contain small amounts of species-specific antibodies, the amounts depending upon the method of immunization employed.

In the case of influenza bacilli, it seems probable that immunization with the S forms also gives rise to species-specific antibodies (anti-R) in addition to type-specific antibodies. When agglutination reactions of S organisms are carried out at 37° only the type-specific antibodies in the S sera are effective, but when the reactions are carried out at 47° the species-specific antibodies can also act. It would of course be hazardous, with present knowledge, to offer an explanation of this, though it may be suggested that at this temperature the capsular substance is dissolved or removed from the surface of the bacteria and the body of the bacterium is exposed.

The cultures were grown in tubes of Levinthal broth, each containing 10 per cent of one of the various kinds of immune serum, or as controls, of normal serum. Transfers of the cultures in these media were made daily. Inoculations from these tubes were also made daily on Levinthal agar plates, in order that the relative number of R and S colonies might be determined.

TABLE III
Interconvertibility of S and R Strains in Antisera

Strain	Type	Number of transfers	Kind of colony after growth in				
			Anti-S serum Type a	Anti-S serum Type b		Anti-R serum	Normal serum
				+41S	+51S		
			+35S			+35R	
35S	a	4	*20S:1R	S	S	S	S
		10	R	S	S	S	S
		20		S	S	S	S
60S	a	5	*1S:12R	S	S	S	S
		7	R	S	S	S	S
		20		S	S	S	S
41S	b					+41R	
		2	*50S:1R			S	S
		4	R			S	S
		10				R	*1S:1R
		13				R	R
51S	b					+51R	
		2			S	S	S
		4			R	S	S
60R		20				S	S
		1				R	R
		2				*10S:1R	R
		5				S	R

* The figures indicate the relative number of colonies of the two kinds on plates.
+ Designation of the particular strain employed in immunization.

From Table III it is seen that when the type-specific strains were grown in media containing the homologous anti-S serum, R colonies appeared on the plate transfers within a short time, much earlier than when growth had occurred in media containing heterologous anti-S serum, anti-R serum, or normal serum.

strains were soluble in bile. Consequently all of the S as well as the R strains were tested as to their solubility. All of the strains were found to be soluble in bile, and no differences in this respect were noted between the S and R strains.

Dissociation of S and R Forms

It has been found that cultures of the newly isolated S form of influenza bacilli are very unstable and that under artificial conditions reversion to the R form frequently occurs quickly and readily.

Transformations may occur when S strains are grown on Levinthal plates or on blood or chocolate agar slants or in Levinthal broth. They may occur when daily transfers are made in broth that promotes luxuriant growth, or when a broth culture is kept in the incubator over a long period of time, or when, after growth has occurred, the broth cultures are kept at 22°C. They may occur when the cultures are kept sealed from the air by vaseline. In S cultures on the surface of blood agar in tubes, transferred every day, R colonies have appeared within a week. Different S strains, however, vary in the readiness and rapidity with which R forms appear. When freshly isolated, the S strains appear more unstable than they do later after they have been repeatedly cultivated. In a relatively stable strain grown through repeated transfers in Levinthal's broth, without replating, transformations do not usually appear until ten or twenty transfers have been made. It has been found, moreover, that the change is delayed in broth cultures in which the conditions are not too favorable for growth, and when the cultures are sealed with vaseline. One such strain has now been kept at 37°C. for 12 months, and subcultures still show that S forms only are present. Other strains, however, kept under the same conditions have not shown the same degree of stability. At 4°C. in Levinthal's broth, under seal, transformations of the S strains have not occurred, but at this temperature the organisms remain alive for comparatively short periods. The longest time such a culture has remained viable has been 15 weeks.

To maintain continuously S strains free of R forms, it has been found necessary to transfer the culture daily on Levinthal agar plates, picking out for each transfer a large typical iridescent colony.

Stryker (38), Griffith, and Reimann (39) have shown that when type-specific strains of pneumococci are grown in type-specific serum transformations may occur. Four type-specific strains of influenza bacilli, two Type a and two Type b strains, have been grown in media containing the two Types of anti-S influenza serum.

occurred in the skin of a rabbit. The rabbit was inoculated with 0.5 cc. of a broth culture, mixed with 1 cc. of vaccine prepared from the Type a strain, 35S. A very marked erythematous lesion, 3.5 cm. x 10 cm., with central necrosis, was produced. Cultures were made daily from the lesion. The first culture contained many R colonies, the second contained S and R colonies, and the third only a few R colonies. All cultures thereafter were sterile. The cultures from the other rabbits of this series gave only R colonies. The S cultures which were obtained from the mouse and the rabbit were both of Type a.

SUMMARY

During the course of a study of different strains of influenza bacilli, fifteen strains have been found to form colonies of a different appearance from that usually considered typical of influenza bacilli. These colonies are smooth, more opaque, and are iridescent in oblique transmitted light. Most of these strains were isolated from patients in whom these organisms seemed to play a pathogenic rôle. When these strains were grown repeatedly on blood agar, other colonies appeared which were smaller, less smooth, less opaque, and not iridescent, and when subcultures were made from these rough colonies, all of the colonies were of this character. Further study of the cultures obtained from these smooth and rough colonies have shown that one is a variant of the other. The strain from the smooth colony has been called an S strain, that from the rough colony an R strain. Certain differences in the morphology of the organisms in the R and S strains have been observed. Of special importance is the fact that the bacteria of the S strains are possessed of capsules. It has also been found that the S strains are somewhat more virulent for laboratory animals than are the R strains.

In the supernatant fluid of broth cultures of S strains, and in the washing fluid of S bacteria grown on agar, there is present a soluble substance which, in the presence of homologous immune serum, gives rise to a precipitate. No reaction of this kind, however, occurs with the cultures of the R strains. By means of cross precipitin reactions, employing antisera against the different S strains, it has been found that the fifteen strains studied may be divided into two distinct immunological groups. Three of these strains belong in one group, Type a, and the remaining twelve in another group, Type b. Seven of the strains studied were isolated from the spinal fluid in cases of menin-

Transformation in the opposite direction, that is, a change of the R form into the S form, has been more difficult to accomplish, just as others have found to be the case with *Pneumococcus*.

Dawson and Avery (40) and Dawson (41), however, have shown that when R strains of pneumococci are repeatedly grown in anti-R serum, transformation into the S form may sometimes occur. Four R strains of influenza bacilli have been repeatedly grown in media containing anti-R serum, using the same technique as in the experiments with S strains described above. In the case of only one of the R strains studied, 60R, did any change from the R to the S form occur (Table III). This strain had only recently been derived from an S strain, while the other R strains studied had been under cultivation as R strains for several months. In this instance, S colonies appeared on the plates in the second transfer, and conversion was complete after five transfers. At no time were R colonies present on the plates inoculated from the control tube which contained normal serum. The S culture in this instance was shown to be of Type 2, as was the original S strain from which the 60R strain was derived.

It seems certain that in these experiments the conversion was influenced by the presence of antibodies, and that the appearance of S colonies on the plates cannot be ascribed to the presence of S organisms in the original culture. The culture employed, however, was not grown from a single isolated cell which would have been necessary to eliminate completely this possibility.

Attempts to convert R into S forms in animals have so far been successful only occasionally.

Experiments have been made with two R strains; one of these had been derived from an S strain several months previously and the other had been derived from an S strain only a few weeks before. Neither of these R cultures produced any soluble specific substance. Both of these strains were repeatedly passed through mice and rats, and were inoculated intracutaneously into rabbits together with killed Type 2 organisms. No successful results have been obtained with the older strain. With the R strain recently isolated, however, two reversions to the S form have occurred. This strain, 60R, is the same one which reverted on passage through media containing anti-R serum. One conversion of 60R took place in a mouse, following an intraperitoneal inoculation of the organisms from 2.5 cc. of a broth culture suspended in 0.5 cc. of broth. The mouse died 36 hours after the inoculation, and S and R colonies were present in the peritoneal and heart's blood cultures. The peritoneal washings of this mouse failed to kill another mouse. Fourteen other mice were inoculated with this strain, and the cultures from these mice contained only R organisms, or they were sterile. The other conversion of 60R

CONCLUSIONS

Strains of influenza bacilli are of two kinds, which have been called S and R. The S strains are distinguished (1) by the appearance of their colonies, smooth surface, large size, opaqueness, and iridescence in oblique transmitted light, (2) by the fact that the individual bacteria are capsulated, and (3) by the fact that they produce a soluble specific substance which is present in culture filtrates and washings of the bacteria. R strains form colonies that are rough and irregular in outline, are less opaque than the S colonies, are of smaller size, and are not iridescent; the individual bacterium possesses no capsules, and these strains produce no soluble specific substance. The S strains are also more pathogenic for animals than are the R strains.

By means of cross precipitation reactions it has been possible to divide the fifteen S strains studied into two distinct immunological types. The same specific types are shown by means of agglutination reactions carried out at a temperature of 37°C. Spontaneous conversion of S strains into the R form occurs in artificial culture media with great readiness. This may be delayed by certain cultural procedures, or may be hastened by growth in media containing type-specific antiserum. Artificial conversion of R strains into the S form has been observed but the changes are carried out only with great difficulty.

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gitis, and all of these strains are of Type b. Agglutination tests performed at 37°C. with these fifteen S strains have revealed the same specific type relationships among the organisms as did the precipitin tests. The R strains on the other hand, exhibit no similar type agglutinations. If the agglutination tests are made at a higher temperature, 47°C., the S strains also fail to show the specific type reactions which occur at 37°C. Certain differences between other biochemical reactions exhibited by the two types of strains have been noted, but it is not believed that they are sufficiently constant to be of great significance.

When S strains are grown on artificial media outside the animal body, they tend to be converted into the R form. The rapidity and the readiness with which this conversion occurs depend on certain conditions, such as the kind of media employed, the temperature at which the cultures are kept, and the atmospheric conditions under which they are cultivated. The rate of conversion is increased when the S strains are grown in media containing anti-S immune serum of the homologous type. On the other hand, conversion of R strains into the S form occurs with much less readiness, and then only if very particular conditions are present. On one occasion conversion occurred when an R strain was grown in a medium containing anti-R immune serum. On two other occasions this same strain changed from the R to the S form during passage through animals. With other R strains it has so far been impossible to bring about this transformation.

These studies indicate that the bacteria belonging in the group *Hemophilus influenzae* exhibit changes in pathogenicity and immunological specificity, which are analogous to those shown by the bacteria of the pneumococcus group. It is important to continue this study, with the technique which has been developed, to include a much larger number of strains. On account of the readiness with which the S strains of influenza bacilli lose their type specificity when grown on artificial culture media, it is important that the organisms be studied as soon as possible after removal from their pathological sources. It is not impossible that many strains lose their specificity immediately after removal from the host, and that the specific immunological differentiation of many strains may, for that reason, be very difficult, if not impossible.

FIG. 5. Bacilli from an R colony of Strain 35R, $\times 1000$. Muir's capsule stain.

FIG. 6. Smear of the peritoneal exudate of a mouse which died $3\frac{1}{2}$ hours after an inoculation of 35S. Wright's stain, $\times 1000$.

FIG. 7. Smear of the peritoneal exudate of a mouse, which died 32 hours after an inoculation of Strain JR. Gram's stain, $\times 1000$. The bacilli have become shaped like globules.

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EXPLANATION OF PLATE 19

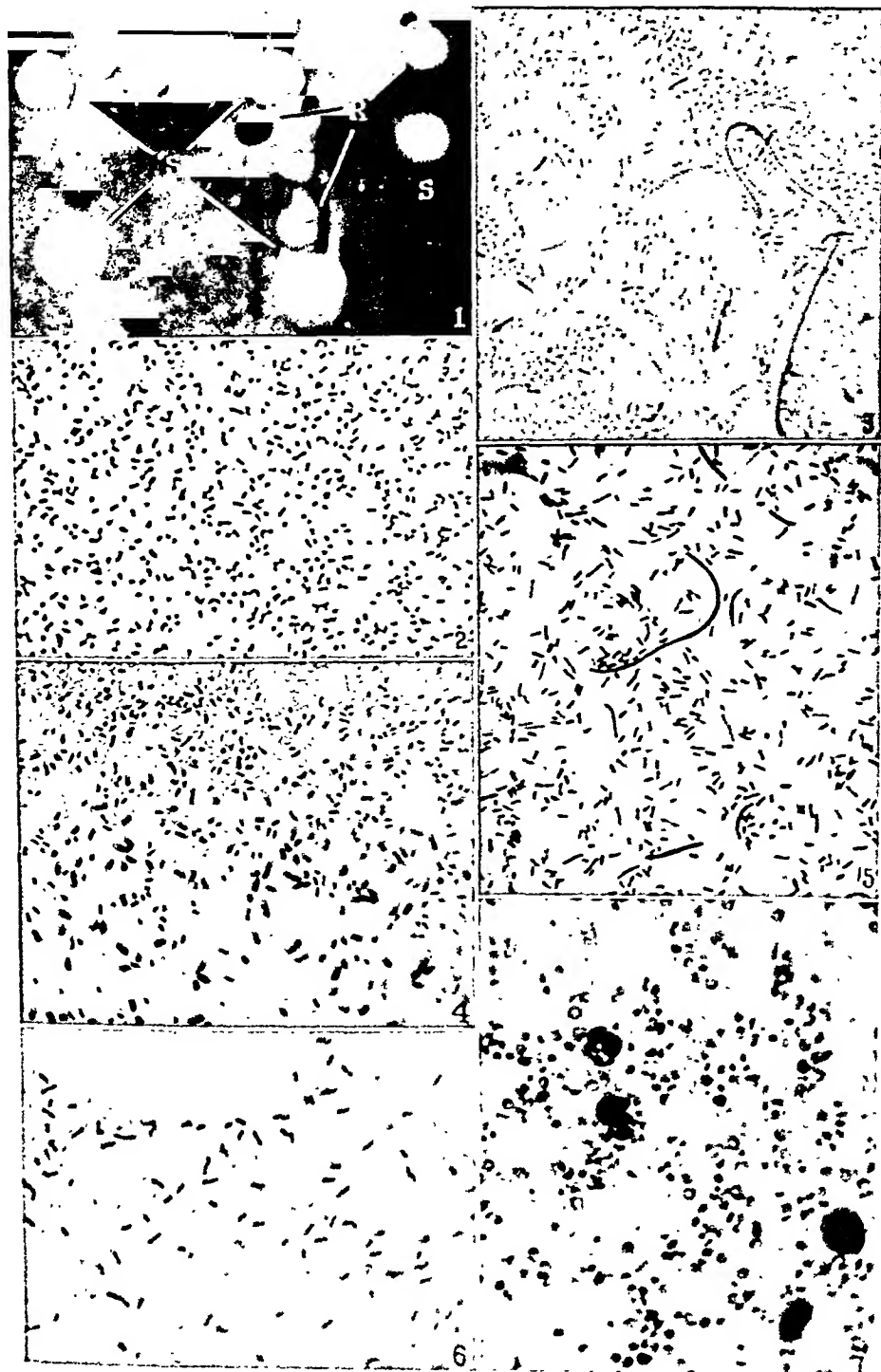
FIG. 1. Plate culture showing S and R colonies of Strain 35, taken by oblique light, $\times 7.5$. Culture 20 hours old. Note the difference in size and deflection of light.

FIG. 2. Bacilli from an S colony of Strain 35S, $\times 1000$. Gram's stain. Culture 20 hours old. Note the uniformity of the rods and compare it with the pleomorphism of the bacilli from an R colony of the same age, Fig. 3.

FIG. 3. Bacilli from an R colony of Strain 35R, $\times 1000$. Gram's stain. Culture 20 hours old. Note the variation in the length of the rods, compare with Fig. 2.

FIG. 4. Bacilli from an S colony of Strain 35S, $\times 1000$. Muir's capsule stain.





it would seem that immunity might be obtained with infected lice, in guinea pigs at any rate, if a method of killing the virus other than heat could be substituted. On the other hand, the concentration of virus for vaccination purposes in lice would be hardly practicable, because lice infected naturally or by the Weigl method must be fed on human subjects for at least 10 days before they are fully virulent, a procedure that requires typhus immune individuals, much labor and a laboratory organization entirely too complex to make this worth considering.

The ideal method of vaccine production of course would be cultivation. The Maitland method is beginning to prove successful in the hands of Nigg and Landsteiner (4), and we, too, have had multiplication of the *Rickettsia* bodies in such cultures, but as far as our own work is concerned, the method has proved so far fraught with so many difficulties and irregularities, and yields amounts of *Rickettsiae* so inadequate that it needs considerable improving before it can be utilized for vaccine production.

We have, therefore, continued with the diet method of producing vaccine for experimental purposes, knowing that thereby we could obtain material sufficiently rich in *Rickettsiae* to permit us a final judgment as to the possibility of producing active immunity with killed virus.

In our preceding papers we have published only upon the results obtained when Mexican *Rickettsia* material was used for vaccination and reinoculation was carried out with European virus. The experiments were carried out in this manner, in the first place, because we were particularly interested in the development of a method of protecting against European typhus, and in the second place, because the reaction of guinea pigs to the living Mexican virus is much more severe than it is to the European variety. In consequence, we thought we would be more likely to be successful in vaccinating against European typhus fever than against the Mexican experimental infection.

In the present communication we wish to report upon an experiment in which we vaccinated against the Mexican variety of the disease.

The vaccine material in this case consisted of peritoneal washings of a guinea pig that was inoculated after the preliminary establishment of experimental scurvy, the animal having lost considerable weight, and showing definite signs of weakness on the day on which he was intraperitoneally inoculated with a suspension of tunica

STUDIES ON TYPHUS FEVER

VII. ACTIVE IMMUNIZATION AGAINST MEXICAN TYPHUS FEVER WITH DEAD VIRUS

BY HANS ZINSSER, M.D., AND M. RUIZ CASTANEDA, M.D.

(From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston)

(Received for publication, January 5, 1931)

In a preceding paper we have described experiments in which partial or complete immunization against European typhus fever was obtained by preliminary inoculations with formalin-killed tunica material containing *Rickettsia* or Mooser bodies. We also described two methods by which a considerable increase in the yield of *Rickettsiae* could be obtained in infected animals, the first one consisting in a preliminary injury of the animals with benzol, the second, and more regularly successful one being based upon the inoculation of animals in the late stages of experimental scurvy.

These methods were resorted to since we had come to believe from the work of Spencer and Parker (1) and of Connor (2), who studied Rocky Mountain spotted fever, and from our own experiments with Mexican typhus fever, that successful active immunization could be hoped for only if considerable amounts of virus were available. Spencer and Parker obtained adequate virus concentrations in Rocky Mountain spotted fever by using the virus obtained from ticks, a single tick often containing as many as a thousand infectious doses.

An analogous method for typhus fever by the use of lice might be successful, but hardly practicable. There is a single experiment of this kind on record in the work of Anderson and Goldberger (3), in which a large number of infected lice were crushed and killed by heating for 30 minutes at 60°C., such suspensions being injected subcutaneously into a monkey. The monkey was not protected by this against subsequent intravenous injection of 3 cc. of defibrinated blood. In view of the experiments we have reported and are about to report,

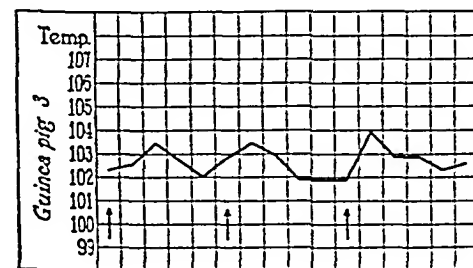
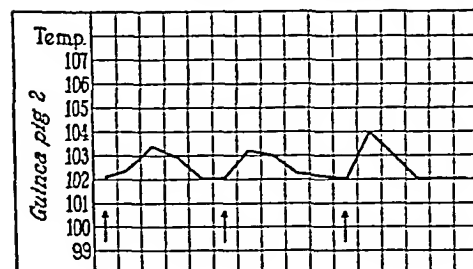
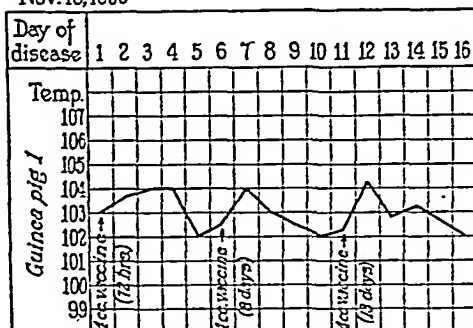
from an active Mexican lesion in which considerable numbers of *Rickettsiae* were present.

This guinea pig was definitely sick on the 5th day after inoculation, reacting in a manner usual to inoculated scorbutic guinea pigs, as described in a preceding paper. In the peritoneal exudate, and in the scrapings from the peritoneum, as well as in the tunica vaginalis, there were large numbers of intra- and extracellular *Rickettsiae*. The material was washed out of the peritoneum with a 0.2 per cent formalin in physiological salt solution. The final suspension, containing cells and many *Rickettsiae*, both intra- and extracellular, represented a concentration of organisms not much inferior to a dilute bacterial culture suspension. The organisms could be found in smears made of the vaccine. It was stored at room temperature for 48 hours before use; after that, in the ice chest. Three male guinea pigs were intraperitoneally treated with this vaccine, 4 cc. of the suspension being injected on Nov. 19th, 25th and 30th. The vaccine, thus, had been in formalin for 3 days, 8 days and 13 days, respectively. This is important as having bearing upon the problem of whether the virus was dead or merely attenuated at the time of injection, a matter of fundamental importance in appraising the value of these experiments. The percentage of formalin is of course of relatively little value in determining its killing power, unless we are dealing with solutions containing equivalent amounts of protein. We cannot, therefore, assume that we worked with killed or attenuated virus—a question of fundamental importance to the significance of the results—merely upon the basis of the concentration of formalin used.

The temperature curves of the three guinea pigs which were intraperitoneally inoculated with the formalinized *Rickettsia* suspension show immediate and sharp rises after each inoculation. We feel confident that these reactions are due to toxicity and not to survival of the virus for the following reasons: The rise of temperature in all cases came immediately, without incubation time, and was followed by rapid return to normal much sooner than this ever occurs in active infection; it was uniform after every inoculation in approximate extent and in time—there being no difference in the general reaction between the first, second and third inoculations, respectively, in all three of the animals; in none of the animals was there, at any time, any sign of scrotal swelling and tunica lesion—a condition which is rarely absent in animals intraperitoneally inoculated with this strain of the disease. We believe for these reasons that, in addition to other things, these experiments show that the *Rickettsia* bodies described by Mooser in this disease possess considerable toxicity. Whether this is in the nature of an excreted poison or of a cellular constituent we cannot state, since we used the entire peritoneal washings in preparing the suspension.

The three animals described were reinoculated, together with their controls, on Dec. 22nd, 33 days after the first and 23 days after the last vaccine injection. The inoculation material consisted of the ground material of the two tunics of a guinea pig in which large numbers of *Rickettsiae* were found. Each animal received about

Nov. 19, 1930



Dec. 22, 1930

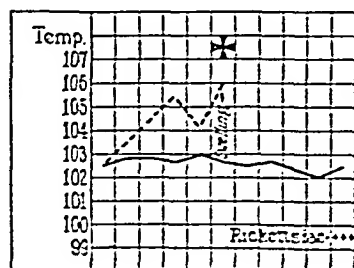
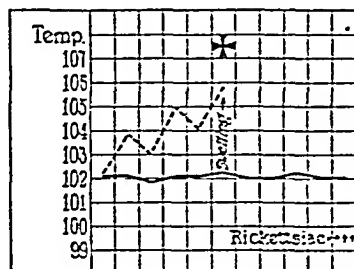
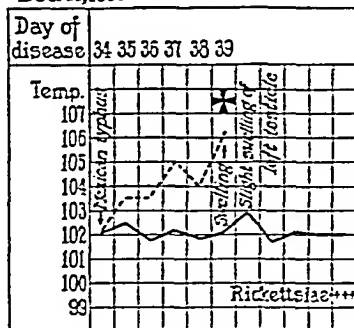


CHART 1. The curves on the left are those of three guinea pigs vaccinated with peritoneal washings of a "diet animal" described in the text. The vaccine was made by treatment with 0.2 per cent formalin and was 3, 8 and 13 days old respectively when injected. On the right are the curves of the same animals inoculated on Dec. 22nd, 23 days after the last vaccination with tunica material from a Mexican typhus animal. The dose was a considerable one and the temperature curves of three controls, inoculated at the same time and with the same material are charted, in broken lines above those of the three immunized animals.

one-tenth of this suspension, therefore one-fifth of the *Rickettsiae* of one tunica vaginalis. The severity of the dose is indicated by the uniform and violent reactions of the controls, which were entirely typical of the severest form of Mexican typhus infection in regard to temperature, scrotal swelling and *Rickettsia* findings.

Of the vaccinated animals, none showed any temperature reaction. The first guinea pig charted, coincident with the rise to 103°F. on the 7th day after inoculation, had a slight enlargement of the left testicle, which was still reducible, but was—for 1 day—obviously abnormal. This completely subsided within 48 hours, but may have represented a localized temporary lesion.

The series of animals is a very small one, but the entire uniformity of the results and their corroboration of experiments published in our preceding papers on vaccination against the European disease with similar materials persuade us that they are worthy of report.

CONCLUSIONS

Guinea pigs can be immunized against Mexican typhus virus by peritoneal injections of formalinized *Rickettsia* material, provided sufficient amounts of the organisms are used. Our results in this respect are analogous to those of Spencer and Parker with carbolized virus of Rocky Mountain spotted fever.

The *Rickettsia* suspensions appear to possess considerable toxicity.

We do not wish to be misunderstood as implying that the results in guinea pigs offer anything more than a demonstration of the principle of active immunization with killed *Rickettsiae*. Application to man will have to be worked out, and preliminary to this, we are now attempting to apply the methods to a limited number of monkeys.

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Materials and Methods

Adult rabbits were used throughout the experiment. They were observed over periods of from 4 to 13 weeks before being used experimentally, during which time numerous complete supravital blood counts, and observations on temperature and weight were made, thus insuring in so far as possible, infection-free stock, and enabling the selection of those animals for experimentation whose blood counts varied least. A postmortem histological survey was made of the usual routine tissues from each animal.

A series comprising twelve animals was studied with injections of protein of three types by three different routes. Two animals received "embryonic extract" intraperitoneally, two intravenously, and one subcutaneously; two received egg albumen intravenously and one subcutaneously; one received normal horse serum intravenously, and one received normal salt intravenously. In addition, splenectomy was performed on two animals, and upon recovery, one received egg albumen intravenously, the other being carried as a splenectomy control without injections of any type. During the experiment, one animal (R 1271*), receiving intraperitoneal injections of embryonic extract, developed a respiratory infection and was discarded.

The protein used for injections was in each case prepared so that each injection carried approximately 50 mg. of protein in 6 cc. of normal salt solution. Embryonic extract (Carrel (7)) was made by crushing one or two 6 to 9 day chick embryos, diluting with normal salt and centrifuging with aseptic precautions to get a clear solution. This solution was made up fresh prior to each injection. Egg albumen was obtained by aseptically aspirating the albumen from a hen's egg and diluting the resultant material with normal salt to constitute a 20 per cent stock solution which was then further diluted just prior to use. The horse serum used was commercial horse serum, and was diluted to proper concentration before each injection.

All intravenous injections were made into the marginal ear vein. In the two animals receiving subcutaneous injections, the amount given to each was divided equally, 3 cc. being injected into the right foot pad and 3 cc. into the right inguinal region. Except in two animals, biopsies were performed with the removal of either the popliteal or inguinal lymph nodes or both, prior to starting the injections. No preliminary biopsy was made in the case of Rabbits R 1264 and R 1158, because it was deemed advisable to preserve the corresponding lymph nodes on both sides in order to control by direct comparison in the same animal the reaction in those nodes that drained the area in which the injection was made.

Injections were made, for the most part, daily, six times per week. Since it has been demonstrated that more than 24 hours is required for the disappearance of horse serum injected into the blood stream (8), there may have been no period during the entire course of these experiments in which the foreign protein was not

* These are serial numbers of the work of the department covering a term of years.

THE INDUCTION OF LYMPHOCYTOSIS AND LYMPHATIC HYPERPLASIA BY MEANS OF PARENTERALLY ADMINISTERED PROTEIN

By BRUCE K. WISEMAN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, January 7, 1931)

Chemical methods of stimulating lymphoid tissue to increased activity have been, in general, unsatisfactory. Some of the reported procedures have given relatively small responses; others have induced a polycellular response in the peripheral blood, and have therefore not been specific for lymphocytes; while still others have depended upon the absorption of bacterial products, and hence the conditions prevailing in disease rather than in physiological states have existed.

The effects of drugs, diets, and physical measures have been reviewed recently by Whitney (1). However, the effectiveness of protein in increasing the lymphocytes in the peripheral blood, when injected intravenously over prolonged periods, does not appear to have been heretofore noted. The acute leucopenia immediately following intravenous administration of protein was observed long ago (2, 3). Moss and Brown (4) found no definite sustained change in total or differential counts after single intravenous injections of 0.1 to 5 cc. of normal horse serum in either sensitized or non-sensitized rabbits. Since their work a considerable literature has accumulated on the blood changes after single parenteral injections of protein, confirmatory but adding very little to the original data. Ehrlich (5) injected staphylococcus of low virulence subcutaneously and found a regional hyperplasia of the lymph nodes and some increase in lymphocytes in the peripheral blood. Injecting killed staphylococci intravenously (6), he obtained an increase in the number of circulating lymphocytes, which reached a maximum at about the 10th and 14th day, and then decreased to normal, although the injections were continued. Hyperplasia of spleen and lymph nodes was observed. A clear differentiation between monocytes and lymphocytes was, however, not stressed.

The observations presented in the present paper were incidental to a study of the effect of chick embryonic extract on the cells of the omental milk spots in rabbits.

present in the circulating blood. Blood examinations, including total red and white cell counts, supravital differential counts, and hemoglobin determinations, were made prior to each injection.

EXPERIMENTAL OBSERVATIONS

Table I summarizes the most important data obtained, including the lymphocyte responses and the weights of the spleens at autopsy. It is evident from this table that all three forms of protein elicit hyperplasia of the spleen, accompanied by a lymphocytosis in the peripheral blood. The greatest response seems to be obtained when the material is given intravenously and probably egg albumen is the most potent of the three substances; a larger series, however, would be necessary to decide this point. It is believed that unless the lymphocyte response is at least 25 per cent above or below the control base line average, it is not justifiable to assume that any effect has been elicited. The fluctuations obtained in the animal receiving only normal saline intravenously (R 1186), and those observed after splenectomy without subsequent injections (R 1187), were well within these limits of normal; the results obtained in the animal receiving embryonic extract intraperitoneally (R 1111) should be regarded as equivocal, though suggestive in view of the results obtained in the other treated animals.

The findings in five of the animals receiving injections of protein (Charts 1 to 5) are typical of this series. All these graphs show an almost specific rise in lymphocytes, which first became evident between the 7th and 10th days after the injections were started. It will be noted that although the average lymphocyte level became constantly elevated, yet there were wide fluctuations within the zone of elevation. That the response and the fluctuations in the lymphocytes are not dependent on the spleen, either through splenic contractions with resultant lymphocytosis, or by the provision of new cells by maturative processes, is indicated by a study of the blood from the splenectomized rabbit (R 1185, Chart 5). In this animal the same phenomena are noted, although the control (Rabbit R 1187) in which splenectomy was performed at the same time but which received no injections showed no noteworthy changes in the blood cells.

Of interest are the occasional neutrophilic peaks or "showers" (see

TABLE I
Changes in Blood Lymphocytes and Weights of Spleens after Parenteral Protein Injections

Rabbit	Material injected	Route	No. of injections	Pre-injection period			Injection period			Percentage increase in lymphocytes	Approximate percentage increase in weight of spleen at autopsy
				Average No. lymphocytes per c.mm. of blood	No. of counts made	Weeks observed	Average No. lymphocytes per c.mm. of blood	No. of counts made	Weeks observed		
R1160	Embryonic extract	Intravenous	45	3,179	13	5	5,599	44	11	76	300
R1157	"	"	19	2,477	21	7	3,911	19	6	58	600
R1111	"	Intraperitoneal	13	3,723	10	4	4,572	18	3	23	400
R1158	"	Subcutaneous	24	3,507	18	7	5,151	24	5	46	None
R1163	Egg albumen*	Intravenous	25	3,208	20	8	6,500	27	5	102	250
R1183	"	"	37	2,436	10	13	4,705	14	5	97	200
R1264	"	Subcutaneous	13	2,356	11	5	3,555	13	5	59	400
R1180	Horse serum	Intravenous	37	3,516	7	13	4,508	14	5	28	300
R1185**	Egg albumen	"	37	2,504	11	13	5,983	13	5	139	—
R1187**	None	—	—	3,067	10	4	2,572	16	6	16***	—
R1186	Normal salt	Intravenous	37	1,793	10	13	2,035	15	5	13	None

* 6 mg. albumen per injection in this animal.

** Splenectomized.

*** Decrease.

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Chart 1, Jan. 24; Chart 3, March 3 and 28; Chart 4, May 2), and monocyte peaks (see Chart 2, Feb. 13; Chart 3, March 28). "Showers" of cells of various types into the peripheral circulation are known to be not infrequent (9, 10).

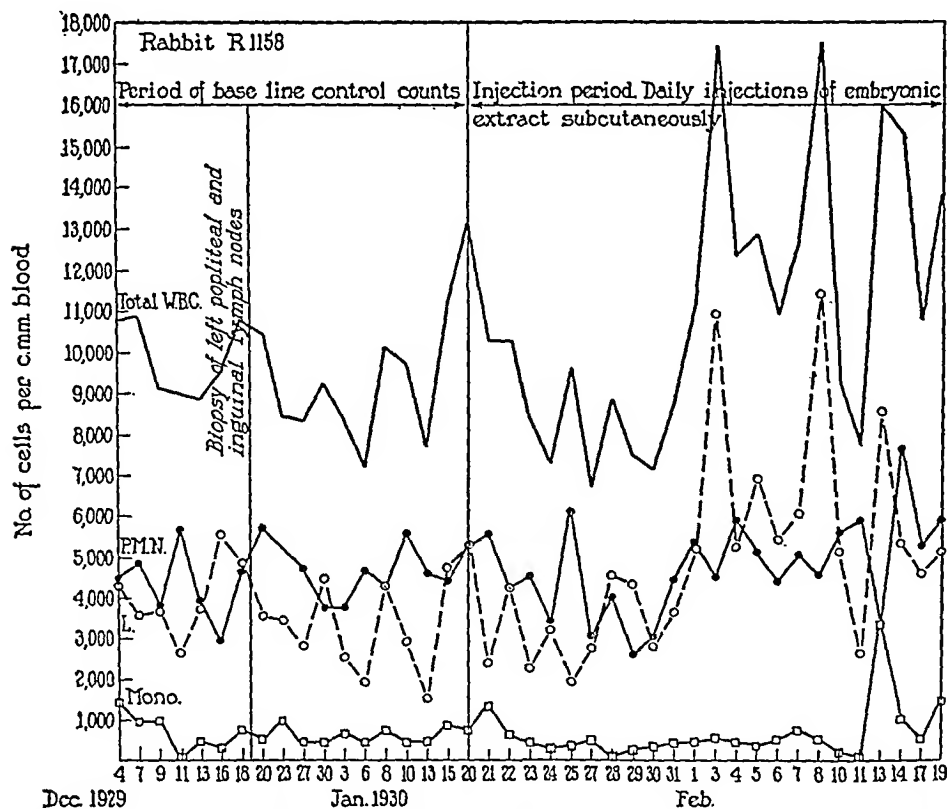


CHART 2. Blood findings in Rabbit R 1158. This animal received 24 subcutaneous injections of embryonic extract, each dose containing approximately 50 mg. solids in 6 cc. normal salt solution. Note that the peaks in total white cells are due almost entirely to corresponding peaks in lymphocytes. The number of monocytes and granulocytes do not show any demonstrable increase except for two small peaks on Feb. 13 and 14.

Chart 6 (Rabbit R 1186), showing the blood counts of a control animal, demonstrates that injections of normal salt gave rise to no changes in blood cells that were not within the limits of normal for the rabbit. The constancy of "zonal levels" (9) is well shown in the chart. This animal received the injections of salt solution concurrently with the protein injections into Rabbits R 1180, R 1183, and R 1185.

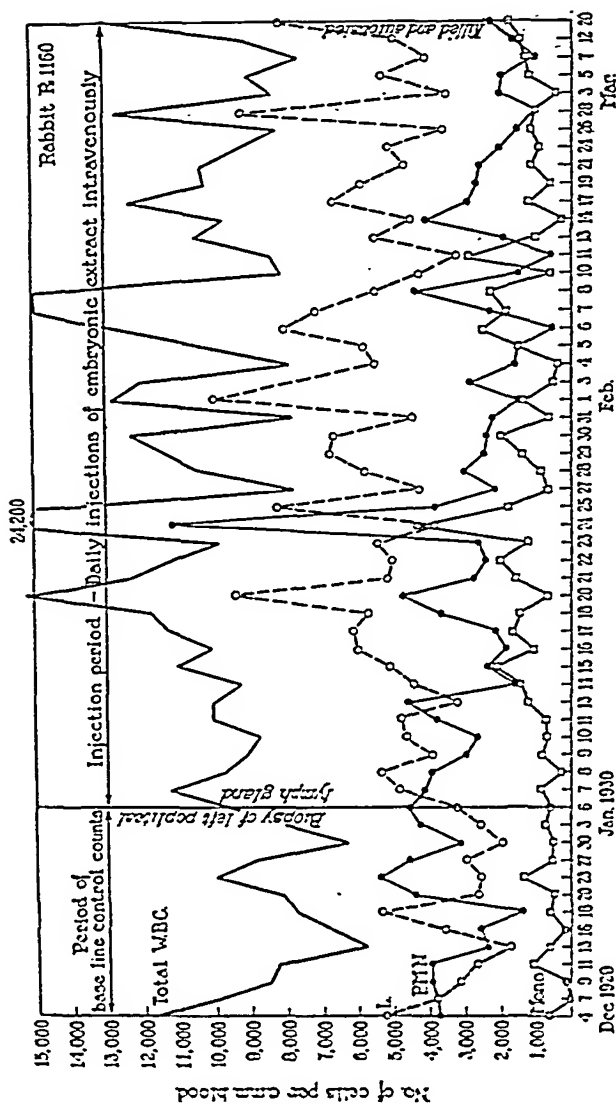


CHART 1. Blood findings in Rabbit R 1160. This animal received on 45 successive days an intravenous injection of embryonic extract, each portion of extract containing approximately 50 mg. solids in 6 cc. normal salt solution. Note the almost specific rise in lymphocytes. One rise or "peak" of neutrophils occurred on Jan. 24. There is also a slightly increased level of monocytes during the injection period.

R 1180 suffered a progressive loss in total red cells during the period of injections and at the time when autopsy was performed, the blood count showed a net loss of approximately one and one-half million erythrocytes per cubic millimeter. No decrease occurred in the other animals. The lack of any increase in eosinophilic leucocytes was

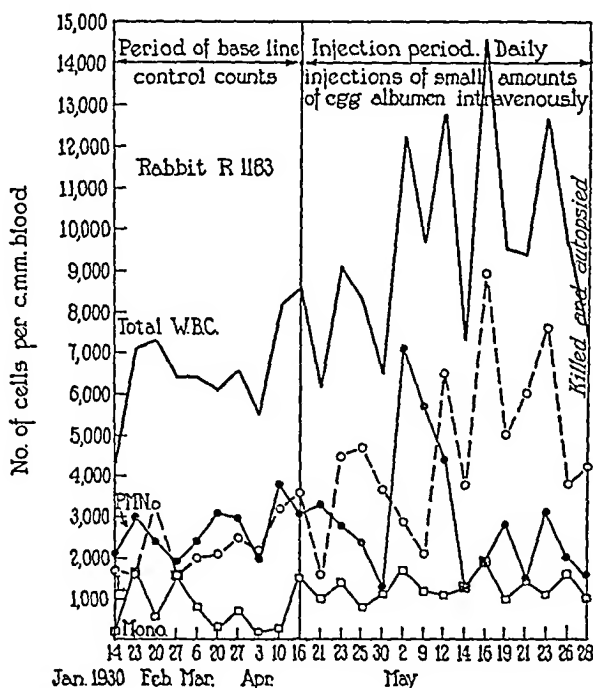


CHART 4. Blood findings in Rabbit R 1183. This animal received 37 intravenous injections, each containing approximately 6 mg. of egg albumen in 6 cc. normal salt solution, on successive days. Note that with the exception of the single neutrophilic peak on May 2, the blood response was entirely limited to the lymphocytes.

very interesting, in view of the fact that there was a profusion of those cells in many of the tissues as proved by histological examination.

An analysis of the histology of the lymph nodes and spleen showed a state of hyperplasia in these tissues. With the exception of the splenectomized animal, in which the nodes removed at biopsy showed some degree of hyperplasia, the nodes of the controls showed no departure from normal. However, in the nodes of the injected animals, removed at autopsy, there was an increase in size of the cortical nodules and a considerable increase in the number of mitotic figures

Qualitative changes in the lymphocytes were quite marked. The supravital preparations showed a great percentage increase in intermediate and large lymphocytes and in cells with strongly basophilic cytoplasm containing great numbers of large mitochondria. Ex-

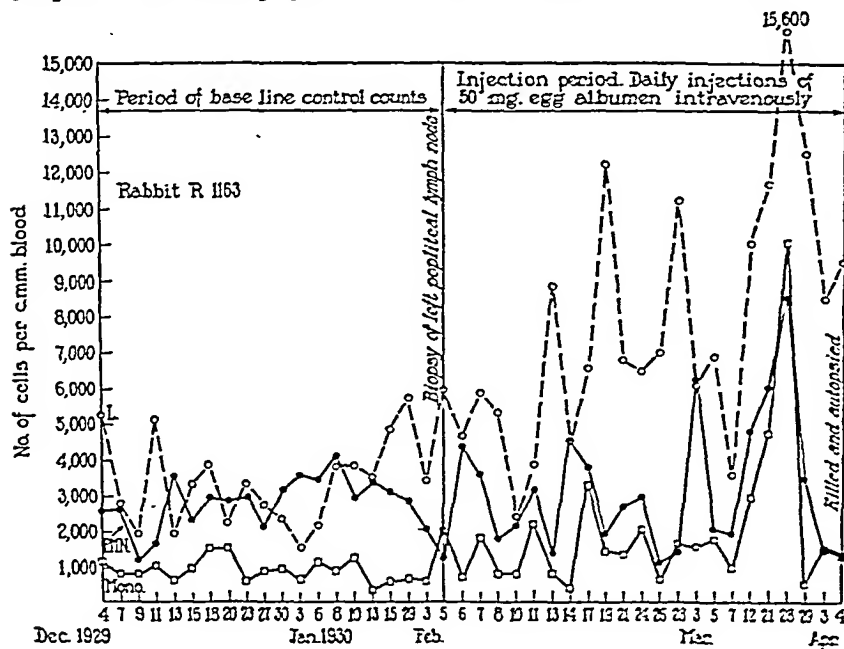


CHART 3. Blood findings in Rabbit R 1163. Twenty-five injections, each of approximately 50 mg. egg albumen in 6 cc. normal salt solution were given this animal on successive days. Note the extraordinarily high count of 16,600 lymphocytes on March 28, and the concomitant high peaks in neutrophils and monocytes. On this day the count was repeated four times at 2 hour intervals and showed (1) that the monocyte peak represented a single "shower" of those cells, (2) that the neutrophils fluctuated between 3,000 and 9,500 cells per c.mm., and (3) that the lymphocytes fluctuated between 6,100 and 16,600. An additional neutrophilic peak was obtained on March 3.

amination of the fixed and stained films confirmed the supravital findings of an increase in types with heavily basophilic cytoplasm. The significance of these changes is dealt with in a succeeding paper.

There was no definite trend in the changes in the red cells, hemoglobin, and eosinophilic and basophilic leucocytes. Rabbit

indicated changes save as concerns the popliteal nodes of Rabbits R 1158 and R 1264, in which the regional lymphatics had been infiltrated with the injection material. These latter nodes were very edematous and it is probable that much of the increases in weight (of 650 per cent and 1,500 per cent, respectively), were due to absorbed fluids.

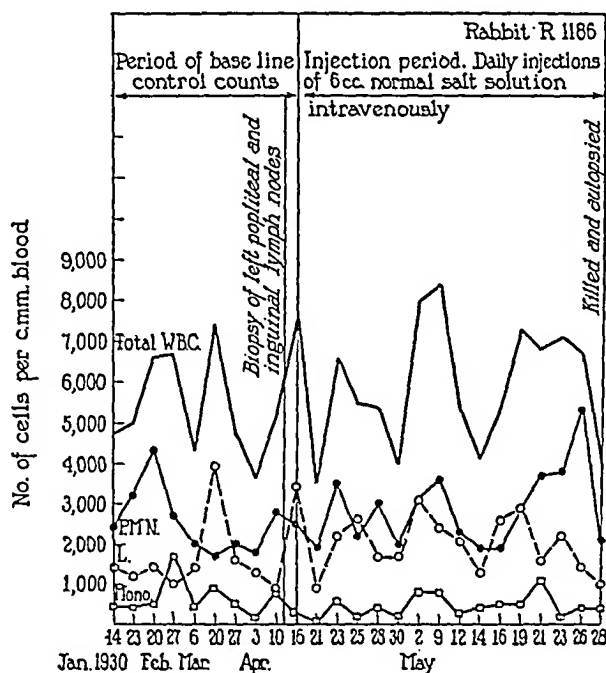


CHART 6. Blood findings in control Rabbit R 1186. This animal received on each of 37 consecutive days an injection containing 6 cc. normal salt solution. Note that all variations in the number of cells are well within the physiological limits of normal for the rabbit.

In the spleen, gross as well as microscopic changes were striking. Grossly, the enlarged Malpighian follicles could be seen through the splenic capsule, closely resembling miliary tubercles. Some were dissected out and were proved by supravital examination to be masses of lymphocytes. Many of the follicles measured approximately 1 mm. in diameter. Microscopically, great hyperplasia with many mitotic figures was found. An increase in eosinophils and plasma cells was also noted.

within the germinal centers of Flemming. The medullary cords were, in some cases, almost obliterated by the encroachment and invasion of lymphoid tissue. Many of the cortical nodules were confluent, resulting, in some cases, in the node assuming the appearance of diffuse hyperplastic lymphoid tissue, as contrasted with the ordinary distinct, corticomedullary architecture of the original normal node from

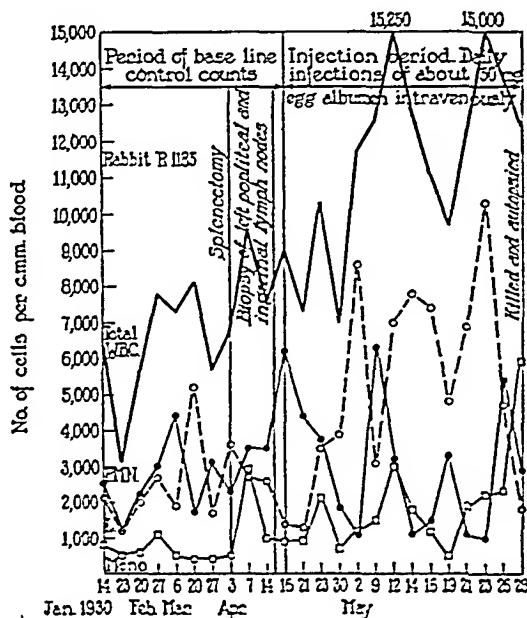


CHART 5. Blood findings in Rabbit R 1185. After recovery from splenectomy, this animal received on each of 37 consecutive days an intravenous injection containing approximately 50 mg. of egg albumen. Note again that the blood changes consist essentially of a sustained rise in total numbers of lymphocytes. Splenectomy control (R 1187) showed no blood changes. This chart also shows an increase in monocyte level during the period of injections.

the same animal. In a few cases, notably in Rabbits R 1163 and R 1183, great numbers of early lymphoblasts were found in the inter-nodular areas, together with patches of cells containing large nuclei with mitotic figures. Increases in eosinophils and plasma cells were also noted. Comparative weights of the popliteal glands at autopsy and the control glands removed at biopsy did not reveal any clearly

The simple method here described for inducing an increase in the lymphocytes of the peripheral blood should be of value for studies into the function and rôle of the lymphocyte. Such studies are in progress.

SUMMARY

Repeated parenteral injections by various routes in a series of twelve rabbits caused an increase of lymphocytes in the peripheral blood varying from 23 per cent to 139 per cent. It seems probable that the degree of response is conditioned upon the type of protein used. At autopsy the lymph nodes and spleen showed hyperplastic changes. The thymus did not participate in the hyperplasia.

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Table I indicates an association between the larger spleens and the lower peripheral lymphocyte counts. Possibly the spleen regulates the level of lymphocytes in the peripheral blood. Barcroft (11) has shown that it acts as a reservoir for red blood cells and Doan, Zervas, Warren, and Ames (12) have described a similar function of the organ in relation to neutrophilic leucocytes in experimental granulocytic leucocytosis after sodium nucleinate.

A great deal of interest centered about the condition of the thymus in the animals. Whether or not the thymus is a true lymphoid organ has long been a matter of debate. Margolis (13) in a recent publication reviews the question and contributes his own observations in a series of autopsies in cases of leukemia. He found no evidence that the thymus participated in the leucemic process.

In the rabbits receiving protein injections no evidence of proliferation of thymic tissue could be detected by microscopic study. It would appear from this finding that the thymus does not take part in the general hyperplasia of the lymphatic tissue, contributing further doubt as to the identity of the small thymic cells with true blood lymphocytes.

Histological examination of the remaining organs of the body showed no noteworthy changes with the exception of an increase in eosinophils in many of the tissues. The number of eosinophilic cells in the bone marrows was especially increased.

At least two explanations are possible of the rôle played by the parenterally administered protein in the determination of lymphocytosis. The substance may act either as a maturative or as a chemotactic factor (14). Since the number of lymphocytes does not increase until the 7th day after the injections are started, it is difficult to believe that a chemotactic effect obtains. On the other hand, it is entirely logical to assume that maturative changes may be stimulated by this agent through a specific functional demand for a specific cell type. Reduction or neutralization of foreign protein which gets through the natural body barriers and enters the tissues may well be one of the functions of the lymphocyte. Regardless of the actual reason for explanation of the phenomena reported in this paper, it seems probable that there is a more intimate relation between certain phases of protein reaction or intoxication in the body and the lymphatic tissue than has heretofore been recognized.

streptococci; while Bell, Clawson and Hartzell (7) have noted scattered glomerular lesions in monkeys after the repeated injection of both hemolytic and non-hemolytic streptococci. Indefinite or entirely negative results have been obtained by LeCount and Jackson (8), by Faber and Murray (9), by Major (10) and by Leiter, all of whom injected streptococci intravenously. Leiter also employed intracardiac injections, to avoid the pulmonary filter, with negative results. Kinsella and Sherburn (11) infected the aortic valves of dogs with *Streptococcus viridans*, and found that those which died within 12 to 14 days showed no glomerular lesion, while those which lived longer than 14 days showed hyaline thrombi and hemorrhages within the glomerulus. Clawson (12) describes focal embolic nephritis following repeated intracardiac injections of *Streptococcus viridans* emulsified with agar. Asch (13) employed the method of injecting bacteria directly into the renal artery in an attempt to produce changes in the kidney. The same method has been used by Winternitz and Quimby (14), by Bloomfield (15), by Pappenheimer, Hyman and Zeman (16), Leiter, and Long and Finner (17). Winternitz and Quimby injected cultures of *B. bronchisepticus* into the renal artery of dogs and obtained a variety of inflammatory lesions, in some instances involving the glomeruli. Both Bloomfield and Leiter used dead cultures of *Streptococcus viridans* with negative results. Pappenheimer, Hyman and Zeman studied the acute changes occurring 24 hours after the injection of living and heat killed hemolytic streptococci into the renal artery of normal and sensitized rabbits. They describe lesions in the glomeruli which were more extensive in the sensitized than in the normal rabbits. Exclusive of the extensive work with diphtheria toxin, there is little evidence to show that the products of bacterial growth, or their "soluble toxins," will cause glomerular lesions in normal animals. Morse (18) used toxic filtrates of staphylococcus with negative results, and Stoddard and Woods (19) employed extracts of streptococcus, staphylococcus and tuberculin without effect. Duval and Hibbard (20) report the reproduction of glomerular nephritis in dogs and rabbits by the intravenous injection of filtered bacteriolysate, obtained from the destruction of hemolytic streptococci injected into the peritoneal cavity of rabbits immunized against this organism. Reith, Warfield and Enzer (21) were unable to confirm these observations. Rich, Bumstead and Frobisher (22) have produced glomerular lesions, with hemorrhage, in rabbits by the intravenous injections of filtrates of cultures of *Streptococcus viridans*. Gray (23) describes lesions, which were not proliferative in character, in the glomeruli of rabbits, following the injection of filtrates from scarlatinal streptococci. Long and Finner produced extensive lesions in the kidneys of tuberculous swine by the infusion of tuberculin into the renal artery.*

*Blackman, Brown and Rake (*Bull. Johns Hopkins Hosp.*, 1931, 48, 74) report the results of convincing experiments, which show that both acute and subacute nephritis, with edema of the tissues, can be produced in rabbits by repeated intravenous injections of an autolysate prepared from Type I pneumococcus. Both the glomeruli and the tubular epithelium were affected.

EXPERIMENTAL ACUTE GLOMERULITIS*

By FRANCIS D. W. LUKENS,† M.D., AND WARFIELD T. LONGCOPE, M.D.

(From the Medical Clinic, the School of Medicine, Johns Hopkins University, Baltimore)

PLATES 20 TO 22

(Received for publication, January 14, 1931)

It is believed by many that acute glomerular nephritis follows as a sequel to some infectious process, the cause of which is usually streptococci, often of the hemolytic type. Observations on the forms of infection associated with acute diffuse glomerular nephritis, and the varieties of bacteria producing these infections have been published elsewhere (1). In another paper (2) there was some discussion of the idea that the onset of the acute attack of glomerular nephritis was dependent, not exclusively upon the acute infection, but, in addition, upon an altered reaction of the body to the infection, an allergic state which might render the cells and capillaries of the kidney peculiarly vulnerable to injury.

The attempts of many investigators to produce glomerular nephritis in animals by the intravenous or subcutaneous injections of bacteria, or by the products of their growth or disintegration, have been only partially and irregularly successful. A series of experiments was therefore devised to determine whether an induced state of hypersensitiveness could be produced, that would increase or alter the response of the kidney cells to large doses of bacterial bodies when they were brought in concentrated form directly in contact with the renal circulation.

The experimental production of glomerular nephritis in animals has been recently reviewed by McNider (3), Leiter (4) and Fishberg (5). The majority of investigators have employed streptococci for this purpose. Ophüls (6) described occasional glomerular lesions after the intravenous injection of rabbits with

* This work was aided by a grant from the Ella Sachs Plotz Foundation.

† Jacques Loeb Fellow in Medicine.

Methods

Two strains of β hemolytic streptococci were used in these experiments. One (SD2) was obtained from the acutely inflamed tonsils of a case of acute nephritis. It produced, by the method previously described (2), a filtrate that was neutralized consistently by convalescent scarlatinal serum in skin tests, and did not produce skin reactions in doses of 0.5 cc. in normal rabbits. The lethal dose of this strain for mice was 0.5 cc. of a 24 hour beef infusion broth culture. The second strain was obtained through the kindness of Dr. Shwartzman, and the filtrate differed from filtrates of many other strains, with which we have worked, in that it caused quite frequently mild or marked skin reactions in normal rabbits. The animals were prepared or "sensitized" by the intradermal injection of 0.2 to 0.5 cc. of a heavy suspension of living β hemolytic streptococci obtained from washing the 24 hour growth from the surface of blood agar slants. At the same time, skin tests were usually made by the intracutaneous injection of 0.5 cc. of filtrate of the same strain of streptococcus, and of 0.2 cc. of heat killed culture. 5 to 10 days after the primary inoculation, a second skin test was performed, and if this proved positive the animals were used for operation on that day. In a few instances a repetition of the procedure was necessary before satisfactory skin reactions were obtained. The normal rabbits, which were used for comparison, were not subjected to preliminary skin tests for fear that this procedure might alter their reaction to the injections of vaccine made into the renal artery.

Cultures of the two strains of hemolytic streptococci, killed by heating at 60°C. for 1 hour, were used for the injections into the renal artery. Vaccines were selected for this purpose because they lacked the power of growth and invasiveness, and because as particulate matter they might be retained in the glomerular capillaries longer than soluble "toxin." With the idea of subjecting the kidney to the possibility of considerable damage, 3.0 to 4.0 cc. of a suspension (usually one 24 hour blood agar slant in 2.0 cc. of saline) of vaccine was injected directly into the left renal artery under aseptic precautions. The animals were anesthetized by ether. The artery was exposed rapidly by blunt dissection without manipulation of the kidney. A 27.5 gauge needle, bent at a right angle, was inserted in the artery. The injection fluid replaced the arterial flow and the kidney became very pale in color. Within a few seconds after the release of the artery and the cessation of the injection, the normal color returned to the kidney. The operation usually required but a few minutes. The method is summarized in detail in Protocol I.

Since it was desired to study any acute changes that might occur in the kidney, the animals were killed from 24 hours to 8 days after injection. Later in the experiments a small number of animals were killed within a few minutes to 24 hours after injection.

Though it has, apparently, been possible at times to obtain scattered glomerular lesions resembling the focal or embolic form of glomerular nephritis in various animals by several methods of experimentation, one must conclude that the histological changes characteristic of the acute diffuse glomerular nephritis in man, or even of an extensive focal glomerular nephritis, have not yet been satisfactorily or regularly produced in animals.

Efforts were first made to produce chronic infections in the paranasal sinuses of goats with hemolytic streptococci with the idea of reproducing conditions that would be analogous to those in man. Probably for mechanical reasons, an infection of the sinuses could not be obtained. A number of preliminary experiments were next made with rabbits. Filtrates of hemolytic streptococci in doses of 10 cc. per kilo were injected intracardially into normal rabbits; and filtrates of hemolytic streptococci, of "Dick Toxin" and of lysates, obtained according to the technic of Duval and Hibbard, were injected into the left renal artery of normal rabbits in doses of from 1.5 cc. to 8.0 cc., the average dose being 3 cc. In sixteen such experiments an examination of the kidneys from 24 hours to 4 days after the injection showed no essential differences between the right and left organs.

After these preliminary experiments it was decided to employ bacterial bodies instead of soluble "toxins," and to study the effect of bacterial bodies upon the kidneys of normal rabbits and of rabbits in which there existed a local streptococcus infection. It has been shown by Zinsser and Grinnell (24), Dochez and Sherman (25), Dochez and Stevens (26), Derick and Swift (30) and by one of us (2) that the skin of rabbits, in which a local streptococcus infection has been produced, acquires the property of reacting to an intradermal injection of the filtrates of hemolytic or green streptococci. The reaction is characterized by an area of erythema which is often edematous. This phenomenon is usually ascribed to the presence of an allergic state. Realizing perfectly that we cannot define this change accurately, and that the rabbits, in the experiments to be reported, were locally infected as well as skin hypersensitive, we will, for convenience sake, term the animals prepared in this manner as "hypersensitive" or "sensitized."

attention, both the tubules and the interstitial tissue were usually affected to some extent. The process was therefore diffuse, inasmuch as the different elements of the kidney structure were involved, but since it rarely happened that all portions of the cortex shared equally in these changes, the lesions often occurred in patches and were thus focal in distribution.

The earliest abnormalities that were observed were seen in the left kidney of a normal rabbit, which was killed 4 hours after the injection of killed culture into the left renal artery.

In this instance a few tubules were dilated and contained hyaline casts. Most of the glomeruli appeared normal, but here and there a glomerulus was seen in which isolated capillaries were filled with hyaline thrombi about which were collected polymorphonuclear leucocytes. Other glomeruli showed an unusual number of polymorphonuclear leucocytes within the capillaries. Similar changes were observed in the left kidney of another normal rabbit killed 8 hours after the injection of killed culture into the left renal artery.

Nine rabbits were killed 24 hours after the intraarterial injection, of which five were normal and four were supposedly sensitized. The left kidneys of two of the five normal rabbits and of two of the four sensitized rabbits were abnormal.

The left kidney of three of these rabbits, two of which were normal and one of which was sensitized, showed very slight changes. These consisted of isolated collections of small round cells about the arcuate vessels, dilatation of occasional tubules which contained hyaline casts, and the presence in isolated glomeruli of hyaline thrombi and collections of polymorphonuclear leucocytes. The left abnormal kidney from the second rabbit, which had been sensitized, showed changes that were similar except that they were much more extensive. A considerable number of glomeruli were involved. There were many in which the glomerular capillaries showed hyaline thrombi, often containing fragmented nuclei. Several glomeruli showed an increase of polymorphonuclear leucocytes in the capillaries, and some showed a collection of a few polymorphonuclear leucocytes directly about the capsule. Many tubules contained hyaline casts. (Fig. 1.)

Thirteen rabbits, eight of which were normal and five sensitized, were killed 48 hours after intraarterial injection. Three of the eight normal rabbits and four of the five sensitized rabbits showed lesions in the left kidney.

Protocol I

Rabbit 383. Weight 2700 gm. Grey brown.

March 19, 1930—At Site A—0.5 cc. killed culture Shwartzman strain intracutaneously. At Site B—0.5 cc. living culture Shwartzman strain (one 18 hr. blood agar slant in 1.2 cc. NaCl) intracutaneously. At Site C—0.5 cc. filtrate Shwartzman strain intracutaneously.

March 20, 1930—A = erythema 1.0 cm.; B = edema, erythema, hemorrhage, necrosis 8.0 cm. x 2.5 cm., C = faint erythematous blush.

March 21, 1930—A = papule 1.0 cm.; B = area 8.0 x 2.5 cm.; C = faint erythema.

March 22, 1930—A = same; B = area 8.0 x 3.0 cm. suppuration; C = negative.

March 24, 1930—At new site A'—0.5 cc. Shw. strain killed culture intracutaneously. At new site C'—0.5 cc. Shw. strain filtrate intracutaneously.

March 25, 1930—A = negative; B = 8.0 cm. x 4.0 cm.; C = negative. A' = papule and erythema 2.5 cm. x 2.5 cm.; B' = erythema 2.5 cm. x 3.5 cm.

March 25, 1930—Injection of 2.5 cc. Shwartzman strain killed culture in left renal artery. 9.43 a.m., ether started; 9.51, abdomen opened; 9.53, left kidney exposed; 9.54½, ligature in place; 9.56, injection started; 9.57½, injection complete, artery released; 9.57½, kidney white; 9.58, color returned; 10.04, ether stopped.

March 26, 1930—24 hours after operation rabbit killed. Right kidney normal; left kidney yellowish with pale mottling. Cut section pale yellowish, cortex thicker than right.

Microscopically.—right kidney normal. Left kidney—practically all glomeruli affected; hyaline thrombi, polymorphonuclear infiltration, pericapsular infiltration, many hyaline casts; collections of small round cells about large arteries. (See Fig. 1.)

Bacterial Stain.—Right kidney—no Gram-positive cocci. Left kidney—Gram-positive cocci plainly seen in a moderate number of glomeruli.

RESULTS

A total of 52 rabbits was used for these experiments, 23 of which had been infected or sensitized, and 29 of which were normal. Of the entire group 25, or 48 per cent, showed acute lesions in the left kidney, and since similar lesions could not be found in the right kidney, which served as a control, it may be concluded that the changes in the left kidney were caused by the vaccine injected into the left renal artery. A description will first be given of the lesions and then the incidence of these lesions will be compared in the infected or sensitized group with those in the normal group.

Although the changes in the glomerular tufts attracted particular

infiltration of the glomeruli were common. Many glomeruli showed the presence of the large cells already described. This gave the appearance of proliferation of the glomerular endothelium or lining cells. Crescentic formations composed of necrotic material or blood, infiltrated with large cells, were frequently seen, and pericapsular infiltration especially by mononuclear cells was marked. (Figs. 5, 6 and 7.)

Twelve rabbits were killed 8 days after injection. Six of these were sensitized and 6 were normal. The kidneys from the normal rabbits showed no abnormal changes. Three of the sensitized rabbits showed left kidneys that were abnormal.

In one of these the changes were extensive and diffuse. Many tubules were dilated and contained hyaline casts, some tubules appeared atrophied. The infiltration of small round cells was diffuse and very marked in places. There was no definite increase in interstitial connective tissue. Almost no normal glomeruli could be found. Many were large and appeared as though composed of large cells with pale nuclei which looked much like the cells of the convoluted tubules but were smaller. Other glomeruli were similar but smaller and the capsule was thickened and cellular. Still others were infiltrated with mononuclear cells, a few so densely that the glomerulus could scarcely be identified as such. Occasionally the infiltration of cells was in crescentic form. There were no hemorrhages. (Figs. 8 and 9.)

The impression that one gains from a study of these sections is of a pathological process which may be detected within 4 to 8 hours after the intraarterial injection of killed streptococci, which progresses rapidly, reaching an acute florid stage within 4 to 5 days, and appears as if undergoing a healing or reparative process by the 8th day. The glomeruli show hyaline thrombi within the capillaries, with necrosis, infiltration of the tufts with polymorphonuclear leucocytes and later small round cells; pericapsular infiltration; the formation of crescents, and an increase in large cells in the later stages of the process. The interstitial tissue shows focal or diffuse infiltration by mononuclear cells and edema. The tubules often show swelling of the epithelium and hyaline casts are numerous, but red blood cells were very rarely seen within the lumen of the tubules.

A comparison of the incidence of the lesions that have been described in the infected, or sensitized group, of rabbits with the normal group, shows that glomerulitis occurs much more frequently in the former. Of the 23 infected or sensitized animals the histological changes of

The intensity and extent of the changes varied considerably. In the sections in which there was least change, there were the same scattered perivascular collections of mononuclear cells and dilated tubules containing casts, such as were seen in the 24 hour sections. There were a few hyaline thrombi in the glomerular capillaries, but many glomerular capillaries appeared empty or were unusually cellular. In some glomeruli there were localized collections of large cells of mononuclear type. In the left kidney from two rabbits the changes were very extensive. There was edema with more or less diffuse infiltration of small mononuclear cells between the tubules. The tubular epithelium was swollen and hyaline casts were numerous. Practically all the glomeruli were involved in the process. The capillaries were often obliterated by hyaline thrombi and the tufts were frequently infiltrated with polymorphonuclear leucocytes. In most of the glomeruli the capillaries did not contain blood. Many of the tufts appeared to consist of necrotic material infiltrated with leucocytes and containing nuclear fragments. There was frequently a pericapsular infiltration of mononuclear leucocytes, but hemorrhages were not seen in the glomerular capsules. A crescentic arrangement of fibrin or necrotic material containing nuclear fragments or elongated cells was very occasionally observed in the glomerular spaces. (Figs. 2 and 3.)

Seventeen rabbits, 9 of which were normal, and 8 of which were sensitized, were killed 3 to 5 days after the intraarterial injection. Fifteen of the total 17 rabbits were killed on the 4th day. More or less extensive lesions were found in the left kidney of all of the 8 sensitized rabbits and very slight lesions in 3 of the 9 normal rabbits.

The changes varied again in this group from scattered lesions to the most extensive and diffuse alterations which occurred in two of the sensitized rabbits. In the kidneys that were least involved the perivascular round cell infiltration was moderate in extent, and there was little change in the tubular epithelium. 10 to 20 per cent of the glomeruli were usually involved. The process appeared much less acute than that studied 48 hours after infection. Occasionally hyaline thrombi were seen but these were rare. More often a collection of large cells was seen in one-half or one part of a glomerulus, filling that part of the capsular space, increasing the size of the glomerulus and sometimes surrounding a hyaline-like center. Other glomeruli were much enlarged, filled the capsular space and were apparently composed of large cells with pale nuclei. It was frequently impossible to distinguish the capillaries. Occasionally there were crescentic bodies about these abnormal glomeruli. Collections of small round cells were sometimes seen about the capsules. (Fig. 4.) The sections from the kidneys showing the extensive lesions were somewhat similar to those examined 48 hours after infection. In one kidney there were definite thrombi in small arteries with infarctions of the cortex. Edema and diffuse infiltration of small round cells was marked. Practically all glomeruli were affected. Capillary thrombosis, necrosis and diffuse

observations show that, when killed hemolytic streptococci are injected into the renal artery of rabbits, acute exudative lesions in the glomeruli and interstitial tissues are much more prone to occur in infected or sensitized animals than in normal animals.

There are other factors, however, which might have some bearing on this differentiation, and which, therefore, had to be taken into account. During the injection of the renal artery there is, of necessity, a momentary cessation of arterial blood flow to the kidney; and it is conceivable that variations in the time of possible renal anoxemia, and of the interference with the blood flow might have some influence in predisposing to the renal lesion.

TABLE III

Relation of Duration of Occlusion of Renal Artery before Intraarterial Injection to Occurrence of Glomerulitis

Duration of arterial occlusion	Total animals	Glomerulitis present	Glomerulitis absent
<i>min.</i>			
0-1	18	7	11
1-2	11	5	6
2-8	23	13	10
Total.....	52	25	27

In Table III, figures are given which show the relation of positive and negative results to the duration of the occlusion of the renal artery. There is no indication that this factor is of importance in determining the occurrence of renal lesions. When the figures are further divided among the infected or sensitized and the normal rabbits, they are quite scattered and show even less evidence that the arterial occlusion has been a factor in the incidence figures. Moreover, the actual occurrence of severe lesions, after very short arterial occlusion, and the absence of lesions, after a very long occlusion lasting 6 to 9 minutes, leads us to disregard this factor.

A study was next made of sections of the kidneys by bacterial stain, to determine how long after the injections the presence of streptococci could be demonstrated in the kidney; and whether the

glomerulitis were present in 17, or 73.9 per cent; of the 29 normal animals they were present in 8, or 27.5 per cent. Further analyses of these changes, according to the strain of streptococcus that was employed, and the severity of the lesions that were produced, are shown in Tables I and II. In the experiments with Strain SD2, the filtrates from which did not cause skin reactions in normal rabbits, the incidence of lesions in the normal rabbits was low: 18 per cent; whereas in the experiments with Strain Shw., the filtrates of which did often

TABLE I

Comparison of the Incidence of Glomerulitis in Normal and "Sensitized" Rabbits

Strain	Normal				"Sensitized"			
	Total	Neg.	Pos.	Pos. <i>per cent</i>	Total	Neg.	Pos.	Pos. <i>per cent</i>
SD2	22	18	4	18	17	6	11	64
Shw.	7	3	4	57	6	0	6	100
Total...	29	21	8	27.5	23	6	17	73.9

TABLE II

Severity of Glomerulitis in Normal and "Sensitized" Rabbits

	Total pos.	Extent of lesion			
		+	++	+++	++++
Normal	8	5	2		1
"Sensitized"	17	9	3	1	4

produce skin reactions in normal rabbits, the incidence of renal lesions was comparatively high: 57 per cent. It was also noted that the total incidence of lesions with the Shw. strain is much higher: 76 per cent + than it is with the SD2 strain: 28 per cent +.

The difference in the figures between the two groups of animals is sufficiently great to be of real significance, and though it will be necessary to repeat these experiments, and possibly to modify them in one way or another, before one can draw definite conclusions, the

a relationship does exist; but it is evident that streptococci may remain in the glomerular capillaries for many hours without giving rise to definite alterations, and the observations show that extensive changes may progress without the demonstrable presence of streptococci.

It was finally necessary to determine whether particulate matter, of approximately the same size as the minute clumps of streptococci in the vaccines, could produce changes when injected into the renal artery of rabbits. For this purpose bismuth oxychloride was selected, since the material was insoluble in physiological salt solution, and since the particles were of approximately the same size as the minute clumps of streptococci in the heated suspensions (Hill (27)).

A preparation, consisting of 5.4 per cent bismuth oxychloride and of 7 per cent acacia in 0.9 per cent NaCl, was used for injection. This suspension was approximately a hundred times as dense as the suspensions of killed streptococci used for injection. 3 cc. of the bismuth suspension was injected into the left renal artery of each of 10 normal rabbits. Four rabbits were killed 48 hours after injection, 13 were killed 4 days and 3 were killed 8 days after injection. In several instances gross infarctions were found in the kidneys, and in a few kidneys a large irregular cell simulating a foreign body giant cell was occasionally found in a glomerulus.

Though particles of bismuth, often in large quantities, could be demonstrated in the glomeruli of eight of the kidneys of these ten rabbits, there were no exudative lesions either in the glomeruli or in the interstitial tissue; and it therefore seemed obvious that a glomerulitis, such as occurred after the injection of killed hemolytic streptococci, could not be produced by particulate matter such as bismuth oxychloride.

DISCUSSION

The experiments described show conclusively that an acute glomerulitis, often simulating very closely certain forms of glomerular nephritis in man, may be produced in rabbits by the injection of a suspension of killed hemolytic streptococci directly into the renal artery. Since only one kidney was affected, the opposite organ appearing normal, an adequate control was available for each experiment.

As the pathological changes, therefore, were limited to one kidney,

persistence of these dead bacteria was related to the presence of glomerular lesions.

In four rabbits, 2 normal and 2 sensitized, dying 5 minutes after injection, numerous Gram-positive streptococci were found in the majority of glomerular capillaries. Two rabbits killed 4 hours after an injection of 1.0 cc. of vaccine showed an increased number of polymorphonuclear leucocytes in the glomerular tufts with moderate numbers of Gram-positive streptococci in the glomerular capillaries.

Into the renal artery of two normal rabbits were injected 3.0 cc. of vaccine from Strain Shw. One rabbit killed 8 hours later showed slight acute lesions in many glomeruli, which contained Gram-positive cocci. The other, killed 16 hours after injection, showed no abnormalities in the kidney and bacteria could not be found

TABLE IV

Rabbit killed; time after injection	Total	Glomerulitis present		Glomerulitis absent	
		Cocci +	Cocci 0	Cocci +	Cocci 0
24 hrs.	9	3	1	1	4
48 "	13	2	4	1	6
4 days	17	4	5	1	7
8 "	13	0	3	0	10
Total.....	52	9	13	3	27

in stained sections. The result of the examinations of the kidneys from the remaining rabbits killed from 24 hours to 8 days after the injection are given in Table IV.

It can be seen from Table IV that, though demonstrable streptococci were by no means constantly present in the kidneys which showed lesions, they were found considerably more often in these kidneys than in the normal kidneys. In this respect there was no essential difference between the kidneys of normal and of sensitized rabbits. When streptococci were found in the abnormal kidneys they were more numerous when the lesions were diffuse than when they were scattered or focal. Although a constant relationship between the presence of glomerulitis and the presence of streptococci in the lesions could not be demonstrated, it must be inferred that such

some unknown reason, they remain entangled in the glomerular capillaries. It is conceivable that the retention of streptococci in the glomeruli may be accounted for by some such phenomenon as that described by Rich and Brown (28) and by Rich (29), who later demonstrated that, independently of an allergic reaction, pneumococci may be clumped, agglutinated and held in the skin when injected intracutaneously into immune rabbits, or injected in combination with immune serum into the skin of normal rabbits. As an analogy to a possible allergic reaction in the kidney, it is important to note that Derick and Swift (30) have described a specific eye reaction to green streptococci in a certain proportion of rabbits rendered sensitive to green streptococci. Julianelle (31) has obtained similar eye reactions in rabbits, which could be elicited by applying to the scarified cornea of rabbits, made allergic to pneumococci by intracutaneous injections, the nucleoprotein or other solution of pneumococcus, from which the acid precipitable and heat coagulable proteins had been removed.

CONCLUSIONS

1. Both focal and diffuse glomerulitis has been produced in rabbits by the injection directly into the left renal artery of suspensions of heat killed hemolytic streptococci.
2. Similar lesions in the glomeruli could not be obtained by the injection of suspensions of bismuth oxychloride into the left renal artery of normal rabbits.
3. The acute glomerulitis occurred in only about one-half of the rabbits employed for the experiments.
4. Glomerulitis was observed much more frequently in rabbits in which an acute localized streptococcus infection had been produced by the intracutaneous injection of living hemolytic streptococci, than in normal rabbits. The occurrence of acute glomerulitis was usually associated with a well marked skin reaction to the filtrates of hemolytic streptococci.

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the process cannot be considered as nephritis, comparable to nephritis in man. The matters, therefore, which were of most concern, were the character of the histological lesions and their mode of origin. It seems clear that the changes in the glomeruli were often associated with the presence of hyaline thrombi in the capillaries and consisted principally of necrosis and exudation. A more careful study of these sections is being made, by special stains, to determine whether or not proliferation of the endothelial cells also occurs. The glomerulitis was often focal in distribution, but at times became so widespread that it involved practically every glomerulus. The histological picture, in many respects, resembled therefore the form of glomerular nephritis that is seen in bacterial endocarditis rather than that encountered after scarlet fever; but unlike the former, the process was not confined to the glomeruli for it affected in addition the tubular epithelium and interstitial tissue. From evidence obtained through a study of sections stained for bacteria, it appears probable that the retention of clumps of dead streptococci in the glomerular capillaries is the direct and immediate cause of these changes. Plugging of the glomerular capillaries by particulate matter alone, however, does not produce these alterations; for in the experiments with bismuth oxychloride no such lesions were found, even though, several days after injection, large amounts of this material were seen in the glomerular capillaries.

It has not been possible to determine all the factors that are responsible for the occurrence of glomerulitis in some animals and its absence in others. The condition of the renal circulation at the time of intraarterial injection, and immediately afterwards, may very well have some bearing on this question; but it has not been possible, as yet, to obtain any definite information as to what this may be. The persistence of bacterial bodies in the glomerular loops seems to be important; and, in combination with this, the only other factor which appears to bear any relation to the variations in the occurrence of the glomerulitis, is the presence of a localized streptococcus infection in the skin of the rabbit, which renders the skin sensitive to the filtrates and dead bodies of the strains of hemolytic streptococci employed. It seems possible that under such circumstances the kidney may be rendered more susceptible than the kidney of normal rabbits to the action of the disintegrated products of dead streptococci, where, for

FIG. 2. Rabbit 218. Normal. 3.0 cc. of heat killed β hemolytic streptococci strain Shw. in left renal artery. Killed 48 hours later. Low power; left kidney showing changes in tubules, interstitial tissue and glomeruli.

FIG. 3. Same as Fig. 2. High power of glomerulus showing hyaline thrombi and pericapsular infiltration by mononuclear cells.

PLATE 21

FIG. 4. Rabbit 302. "Sensitized" to β hemolytic streptococci Strain SD2. 3.0 cc. heat killed β hemolytic streptococci SD2 in left renal artery. Killed 4 days later. Low power; left kidney showing focal infiltration by small round cells and changes in glomeruli.

FIG. 5. Rabbit 275. "Sensitized" to β hemolytic streptococci Strain SD2. 3.0 cc. heat killed β hemolytic streptococci Strain SD2 injected into left renal artery. Killed 4 days later. Low power; left kidney showing diffuse changes in tubules, interstitial tissue and glomeruli.

FIG. 6. Same as Fig. 5. High power; glomerulus showing hyaline thrombi with necrosis and pericapsular infiltration of small round cells.

PLATE 22

FIG. 7. Same as Fig. 5. High power; glomerulus showing crescentic formation.

FIG. 8. Rabbit 304. "Sensitized" to β hemolytic streptococci Strain SD2. 3.0 cc. heated killed β hemolytic streptococci SD2 injected into left renal artery. Killed 8 days later. Low power; left kidney showing extensive diffuse infiltration of interstitial tissue, changes in tubules and glomeruli.

FIG. 9. Same as Fig. 8. High power view of glomerulus.

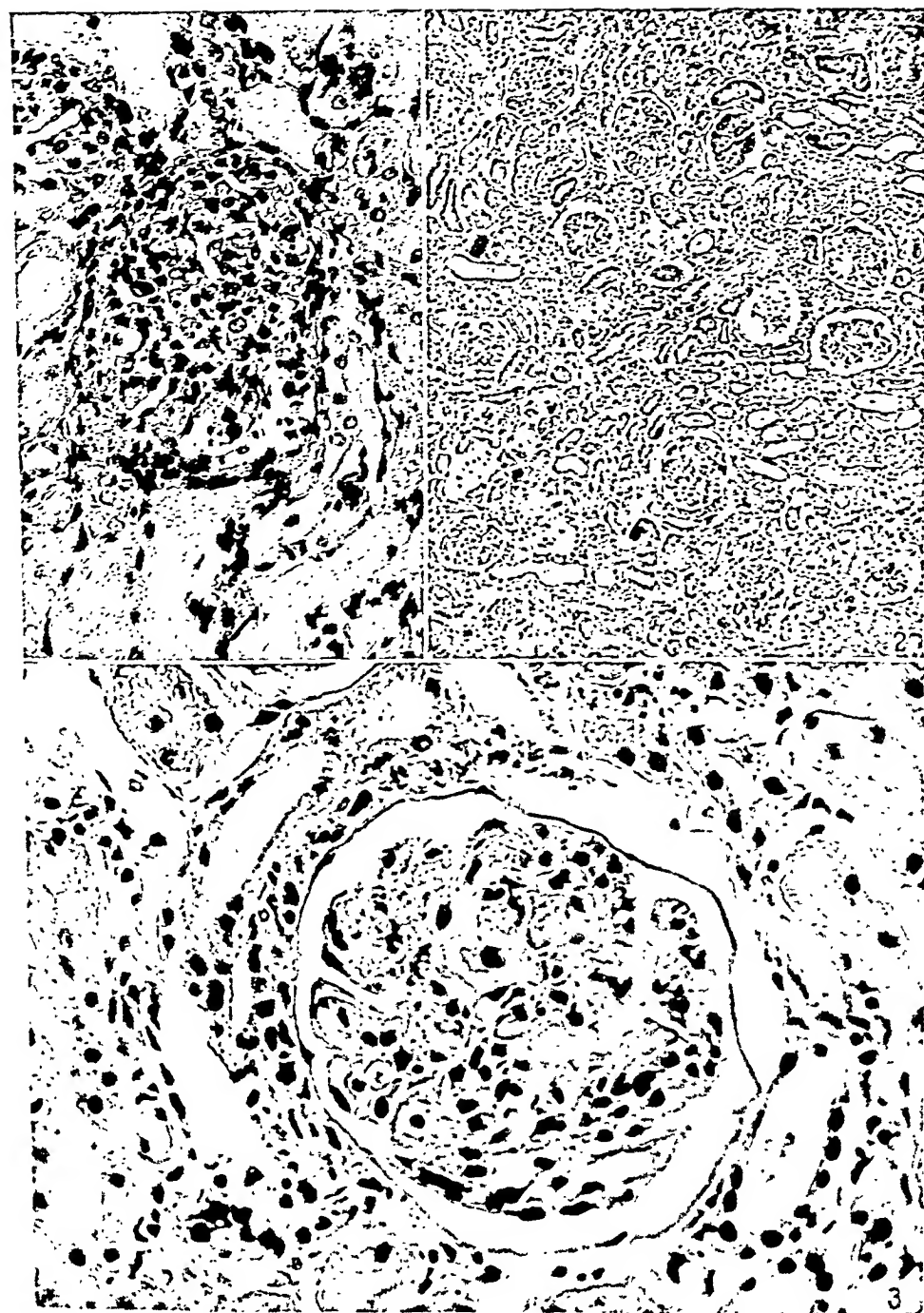
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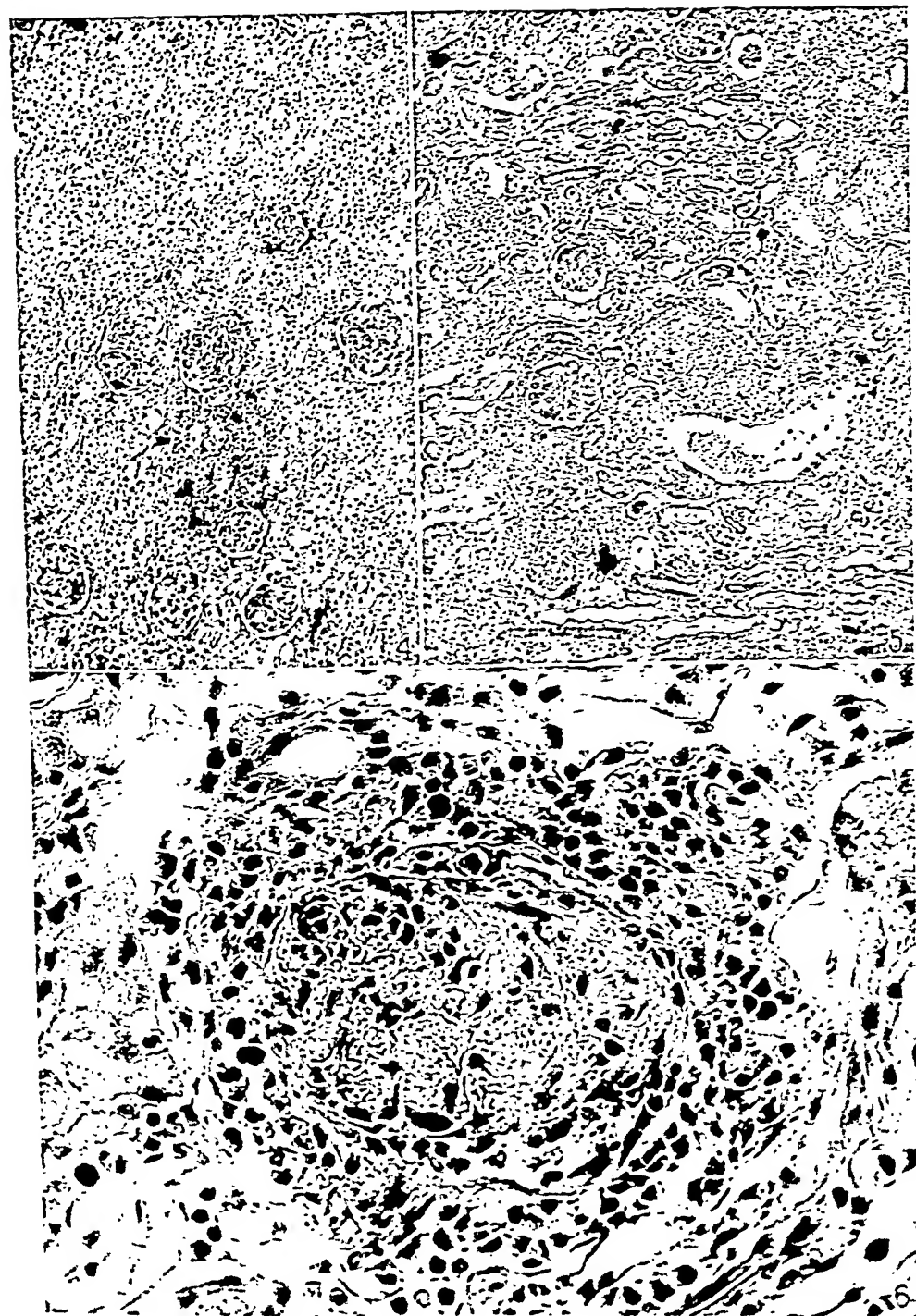
EXPLANATION OF PLATES

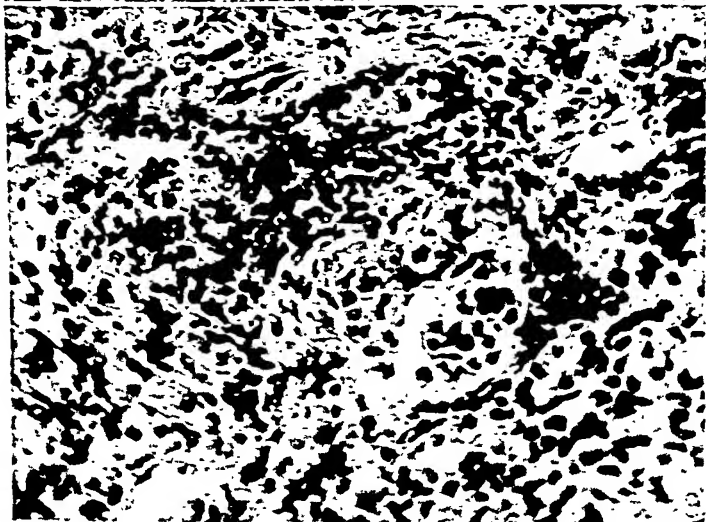
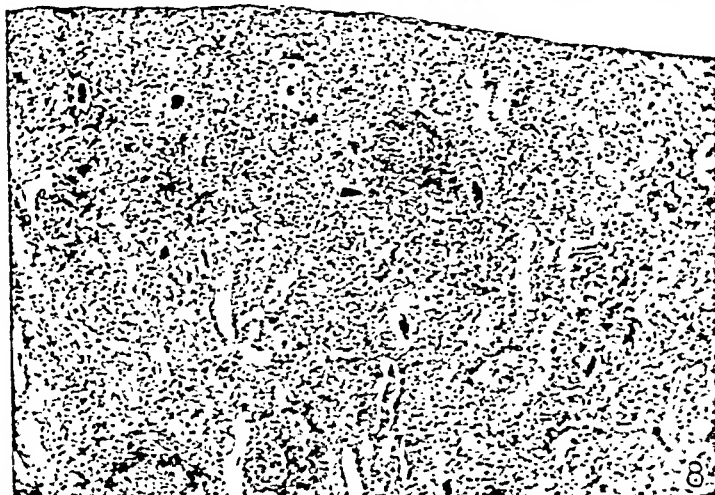
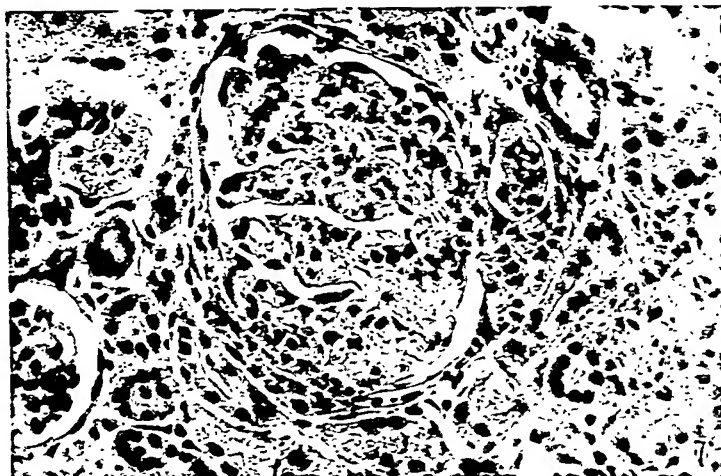
PLATE 20

FIG. 1. Rabbit 383. "Sensitized" to Strain Shw. β hemolytic streptococci. 2.5 cc. heated killed Shw. into left renal artery. Killed 24 hours after intraarterial injection. High power; left kidney showing acute changes in glomerulus with pericapsular infiltration.









After removal of the sediment from the concentrated solution, dilute acetic acid was added to test for protein. When no precipitate was formed by the small amount of acid, a sample was removed and treated with increasing amounts of acid. When about one-half volume of glacial acetic acid was added, a heavy white precipitate was formed. Solutions of this precipitate dissolved in H_2O and adjusted to neutrality, gave strong Molisch reactions, reacted in high dilutions against antiyeast sera, and gave definite though weak reactions against Type II antipneumococcus serum. After observing the reactivity of the material obtained by treatment of the small sample with large amounts of acetic acid, the original batch was treated in the same way. The precipitate was redissolved in H_2O , adjusted to pH 7.4, and used as the stock solution of yeast antigen in the subsequent experiments.

Preparation of Pneumococcus Antigen.—Heavy suspensions of washed pneumococci in salt solution were frozen and thawed until the cells were thoroughly broken up. The sediment was removed by centrifugation. The supernatant fluid was treated several times with dilute acetic acid to remove the greater part of the dissolved protein.

Anaphylaxis Tests.—The guinea pigs (275 to 300 gm.) were sensitized by intravenous injection of 1.0 cc. of serum. A rest period of 24 hours was given between the sensitization and the intravenous injection of the test antigen. None of the test antigen solutions were primarily toxic in control tests on non-sensitized (normal) guinea pigs.

Antiyeast Serum vs. Pneumococcus Antigen

There is considerable variation (1) in the antipneumococcus (Type II) potency of the antisera of different individual rabbits immunized with yeast. Some of them are only weakly reactive but the most potent ones agglutinate and protect against Type II pneumococci as well as the average antiserum obtained by immunization with the Type II pneumococci themselves. An antiyeast serum of high order of potency in respect to agglutination and protection against the Type II bacteria is best fitted for tests of anaphylaxis and precipitation of solutions of semi-purified carbohydrate extracted from the pneumococci. Table I illustrates its use to test the type specificity of the inter-reactions of antiyeast serum and pneumococcus antigen.

The data of Table I show that the reactions of the antiyeast serum were as specific for Type II pneumococci as concerns anaphylaxis and precipitation against the soluble antigen as had been previously (1) found, using agglutination and protection against the whole bacterial cells. That is, the antiyeast serum possessed the capacities to precipitate and to sensitize guinea pigs against the Type II antigen but was devoid of the same capacities for reaction with the antigens derived from Types I and III.

STUDIES ON IMMUNOLOGICAL RELATIONSHIPS AMONG THE PNEUMOCOCCI

V. ANAPHYLAXIS AND PRECIPITATION BETWEEN ANTIGENS AND ANTISERA OF YEAST OF TYPE II PNEUMOCOCCI

BY JOHN Y. SUGG AND JAMES M. NEILL, Ph.D.

(From the Department of Bacteriology and Immunology, Vanderbilt University
Medical School, Nashville)

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Previous papers (1, 2) reported evidence of immunological relationship between a variety of *Saccharomyces cerevisiae* (yeast) and the Type II variety of *Diplococcus pneumoniae* (pneumococcus). The relationship, apparently dependent upon S-anti-S reactions, was evidenced by the immunological reactions of agglutination and of passive protection in which the test antigens were the whole cells of the microorganisms. In the present paper the relationship between the same two microorganisms is evidenced by the immunological reactions of anaphylaxis and precipitation in which the test materials were solutions of antigen extracted from the microbial cells. Most of the protein at least had been removed from these test solutions and there is every reason to believe that the antigen responsible for the anaphylaxis and the precipitation was carbohydrate.

EXPERIMENTAL

Methods

Preparation of Yeast Antigen.—Dried (Fleischmann's) yeast was added to 0.05 N HCl and boiled 1 hour with frequent stirring; after storage overnight in the ice box, the yeast cells were removed by centrifugation. Acetic acid was added to the supernatant fluid and the protein precipitate removed by centrifugation. The supernatant fluid was precipitated with ten volumes of alcohol and the precipitate dissolved in H₂O. Dilute acetic acid was added to the solution and the supernatant fluid was again precipitated in alcohol. The H₂O solution of the precipitate was treated with dilute acetic acid, and, after removal of the protein precipitate, the solution was made neutral and concentrated *in vacuo* over phosphoric anhydride to one-fourth its volume.

The data (Table II) show that the yeast antigen was reactive with the Type II antiserum but not with the Types I and III antisera. It is evident, however, that while the reactions were definite* the degree of reactivity of the pneumococcus (Type II) antiserum with the yeast antigen is not at all comparable to the high degree of reactivity (Table I) of the pneumococcus (Type II) antigen with the antiyeast serum. For example (Table II), the anti-Type II serum precipitated the yeast antigen only in 1:5 dilution in comparison to the precipitation of 1:3200 dilution by the antiyeast serum, and animals sensitized with the anti-Type II serum gave only sublethal reactions* to doses of yeast antigen over 300 times as great as those causing acute death

TABLE II

Reactivity of Yeast Antigen with Anti-Type II Serum and Its Non-Reactivity with Antisera of Types I and III Pneumococci

Antiserum	Precipitation	Anaphylaxis	
		Dose	Reaction
	<i>dilution</i>	<i>cc.</i>	
Type I	0	2.0	None
Type II	1:5	2.0	Sublethal*
Type III	0	2.0	None
Antiyeast	1:3200	0.0006	Death
		0.0002	Sublethal

of animals sensitized with the homologous antiyeast serum. In contrast to this marked disparity between the degrees of reactivity of the antiyeast and of the anti-Type II sera in tests with the yeast antigen, the results in Table I showed that some of the more potent antiyeast sera possess a degree of reactivity with the Type II pneumococcus antigen approximately equal to that of an anti-Type II serum itself. A

* Although no immediate deaths were obtained by injection of 2.0 cc. doses of yeast antigen in guinea pigs sensitized with Type II antiserum, pronounced sublethal reactions were obtained with all of the six animals tested. Death would probably have followed the use of larger test doses but it was impossible to try these because of the limited amount of antigen at our disposal. In view of the complete absence of reaction on the part of the animals sensitized with the Types I and III antisera, the pronounced reactions of the anti-Type II sensitized animals can be taken without question as evidence of true anaphylaxis.

The relationship between the antibody in the antiyeast serum and the antigen of Type II pneumococci is made more significant by quantitative comparison of the results with the Types I and II antigens. In tests against its homologous antiserum the solution of the Type I antigen was precipitated in dilutions as high as 1:1600, and doses of it as small as 0.01 cc. caused prompt death of sensitized guinea pigs; but when tested against antiyeast serum it failed to be precipitated in any dilution and likewise failed to cause anaphylaxis of sensitized guinea pigs even when the dose was 200 times the M.A.D. required with the homologous antiserum. In contrast, the Type II antigen was almost as reactive against the antiyeast serum as against its own antiserum: for example, precipitation of the Type II antigen

TABLE I

Reactivity of a Potent Antiyeast Serum with Type II Pneumococcus Antigen and Its Non-Reactivity with Similar Antigens from Types I and III

Pneumococcus antigen	Precipitation		Anaphylaxis			
	Homologous antiserum	Antiyeast serum	Sensitization with homologous antiserum		Sensitization with antiyeast serum	
			Dose	Reaction	Dose	Reaction
	<i>dilution</i>	<i>dilution</i>	<i>cc.</i>		<i>cc.</i>	
Type I	1:1600	0	0.01	Death	2.0	None
Type II	1:800	1:400	0.01	Death	0.02	Death
Type III	1:50	0	0.5	Death	2.0	None

occurred in about as high dilution with antiyeast as with anti-Type II serum and the doses required for anaphylaxis were only twice as great for guinea pigs sensitized with antiyeast as for those sensitized with anti-Type II serum. (The quantitative contrast is not so great with the Type II antigen but this is due to the lower order of potency of the anti-Type III serum.)

Yeast Antigen vs. Antipneumococcus Sera

The solution of semi-purified carbohydrate from yeast was tested against the different types of antipneumococcus sera. Each of the antisera selected was of a high order of potency against antigen derived from the homologous type of pneumococci. The results are summarized in Table II.

sitize: the antiserum from No. 1 sensitized sufficiently to give prompt death with doses of 0.02 cc. while in extreme contrast the antiserum from No. 4 did not sensitize sufficiently to give even sublethal reactions to doses 100 times as great. These differences in respect to

TABLE III

Differences in Respect to Degree of Reactivity with Pneumococcus Antigen Exhibited by Antisera Possessing Equal Degrees of Reactivity with the Yeast Antigen

Rabbit	Precipitation		Anaphylaxis			
	Yeast antigen	Pneumococcus antigen	Yeast antigen		Pneumococcus antigen	
			Dose	Reaction	Dose	Reaction
	<i>dilution</i>	<i>dilution</i>	<i>cc.</i>		<i>cc.</i>	
1	1:3200	1:400	0.0006	Death	0.02	Death
			0.0002	Sublethal	0.01	Sublethal
2	1:3200	1:100	0.0006	Death	1.0	Death
			0.0002	Sublethal	0.5	Sublethal
3	1:3200	0	0.0006	Death	2.0	Sublethal
			0.0002	Sublethal	1.0	None
4*	1:3200	0	0.0006	Death	2.0	None
			0.0002	Sublethal		

* Antiserum 4 was not entirely devoid of anti-Type II antibodies; they were demonstrable by passive protection tests against small doses of Type II culture, which apparently is an especially delicate test method for antisera only weakly reactive against Type II antigen.

potency against the pneumococcus antigen are especially important because observed in antisera that showed no significant difference in degree of reactivity against the antigen homologous to that with which the animals were immunized. Differences of this sort in the antisera of individual rabbits emphasize the difficulty of obtaining a complete expression of the antigenic properties of cells containing a variety of components unless a large number of individual animals are included in the immunization.

COMMENT

The data present two points of interest. The first is the inter-reactions of precipitation and of anaphylaxis by solutions of semi-purified

part of this difference is probably due to the fact that the antiyeast serum used in Table I was selected on the basis of its antipneumococcus potency. However, the same difference was observed in the previous study (1) on the basis of agglutination with the antisera from a large number of rabbits, and hence, one can conclude that as a rule the antisera of animals immunized with yeast are more reactive with the pneumococcus antigen than the antipneumococcus sera are with the yeast antigen. In contrast, in the inter-reactions of Type II with Subgroup II pneumococci, the Type II antisera are more reactive with Subgroup II antigen (3) than the Subgroup II antisera are with Type II antigen.

*Differences in Respect to Reactivity with Pneumococcus Antigen
Exhibited by Antiyeast Sera of Equal Degree of
Reactivity with Yeast Antigen*

The reactions of antiyeast serum against pneumococcus antigen presented in Table I were obtained with an antiyeast serum selected as particularly potent in respect to antipneumococcus reactivity. However, the antisera obtained by immunizing different rabbits with yeast show marked differences in respect to the degree of reactivity with Type II antigen. In the preceding study (1), this was evidenced by the wide difference in the capacities of different antiyeast sera to agglutinate and protect against the Type II bacterial cells. In the present investigation the same sort of differences were manifested in the capacities of individual antiyeast sera to precipitate *in vitro* and to sensitize *in vivo* against solutions of the Type II antigen. This point is illustrated by a summary (Table III) of the precipitation and anaphylaxis tests of the antisera obtained from four rabbits.

It is evident (Table III) that the potency in respect to reactivity with the yeast antigen can be accepted as uniformly good in the antisera from all four of the rabbits: all of them precipitated the yeast antigen in dilution of 1:3200 but not in 1:6400 and they also sensitized guinea pigs sufficiently to give rise to prompt death upon the subsequent injection of 0.0006 cc. of antigen solution though not to doses of 0.0002 cc. If the four antisera are compared, however, with respect to reactivity with the pneumococcus antigen, marked differences are evident. Whereas, the antiserum from Rabbit 1 precipitated the pneumococcus antigen in dilution of 1:400 and that from Rabbit 2 in dilution of 1:1000, the antiserum from No. 3 and from No. 4 failed to precipitate it in any dilution and even when not diluted at all. Similarly pronounced differences were evident in the capacities to sen-

Pronounced differences in respect to reactivity with the pneumococcus antigen were found between individual antiyeast sera that were of equal potency in respect to reactivity with the yeast antigen. This fact emphasizes the desirability of the use of antisera from a large number of individual animals in the study of microbial inter-relationships.

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carbohydrate antigen extracted from Type II pneumococci and from yeast. Cross-reactions of anaphylaxis were to be expected from the immunological relationship previously (1, 2) demonstrated by agglutination and protection. But, production of anaphylaxis by injection of pneumococcus material in animals sensitized with antiyeast serum is of more than ordinary interest because of the wide genetic distance between the microorganism (ascomycete) used for the immunization and that (schizomycete) used for the shock antigen.

The second point of interest is the pronounced difference in respect to reactivity with the pneumococcus antigen found between individual antiyeast sera that were of equal potency in respect to their reactivity with the yeast antigen. Similar observations were made in our previous studies (4, 5) on pneumococci related to the usual Type III, the antisera of individual rabbits differing widely in their reactivity against the related bacteria even when they showed no differences in reactivity against the homologous microorganism. Thus, although it was easy enough to obtain antisera potent against the dominant antigen of the homologous microorganism, it was necessary to immunize a large number of animals in order to obtain antisera representing a complete expression of all the different antigenic constituents included in the microbial cells used in the immunization. This is an important factor to consider in all studies of microbial inter-reactions. Although the antisera obtained by immunizing one or two animals may be adequate for studies dealing with the dominant antigen of the homologous microorganism, they cannot be depended upon in studies dealing with the antigens of related microorganisms. For the latter, a large number of animals should be immunized, for in some instances only the most responsible individuals will yield antisera that reveal all of the immunological relationships.

SUMMARY

This paper reports inter-reactions of anaphylaxis and precipitation between antigens and antisera derived from the Type II variety of pneumococcus and from one variety of yeast. That the reactions occurred only with Type II and not with Types I and III is proof that the pneumococcus antigen responsible for the anaphylaxis of the antiyeast sensitized animals was the type-specific carbohydrate (S).

the differences and variability of the response of individuals to exposure to pneumococci. The results of these studies are described in the present paper.

Technique

Clinical observations and nose and throat cultures were made on four separate groups of normal people. The first group consisted of 33 adults connected with The Rockefeller Institute, together with six children and adults in their respective families. These individuals, living under relatively similar environmental conditions, were chosen to represent a typical cross-section of an urban population. Each person was questioned at 2 or 3 day intervals to ascertain the presence of coryza, sore throat, headache, malaise, or fever, and at the same time was instructed to report voluntarily whenever such symptoms were present. As a rule, bacteriological cultures of the nasal passages and throats of these people were made every 7 days; during periods of illness, however, a report was made and one to three cultures were taken daily. Individuals of this group have been under observation for periods of 7 months to 3½ years. The second group consisted of 19 children at the New York Foundling Hospital. These boys and girls, 5 and 6 years old, lived in two relatively isolated units comprising playroom, dining room, and bedroom, and were each under the care of one special nurse. Nasal passage and throat cultures were made bimonthly from November, 1929, to March, 1930. The third group of individuals consisted of 25 children, aged 3 to 54 months, living in their respective homes in New York City. They were brought to the clinic of the New York Nursery and Child's Hospital about once a month for physical examination. On these occasions, from February to June, 1930, cultures were made from their nasal passages and throats.¹ The fourth group consisted of 22 children, aged 5 and 6 years, attending the Bethlehem Day Nursery in New York City. These children were studied during May and June, 1930.

In making a culture from the nasal passages the individual was requested to exhale forcibly and a sterile swab was then passed into each nasal orifice. In making a culture from the throat a swab was passed over the entire posterior pharyngeal wall and the surface of the tonsils. The swabs were then streaked over the surfaces of media contained in 10 and 15 cm. Petri dishes.

Media were prepared according to the method of Avery, Chickering, Cole, and Dochez (2). The best grade of fresh beef was employed and heating was reduced to a minimum. Slants and plates made with fresh agar plus 5 per cent whole, citrated, rabbit blood were kept moist and were used within a few hours after their preparation.

The inoculated plates were incubated in a moist atmosphere at 37.5°C. for 18 hours, after which the resulting bacterial growth was classified into the relative

¹ The authors wish to express their thanks to Dr. Dorothea Moore for assistance which made possible the observations on this group of children.

THE EPIDEMIOLOGY OF PNEUMOCOCCUS INFECTION

THE INCIDENCE AND SPREAD OF PNEUMOCOCCI IN THE NASAL PASSAGES AND THROATS OF HEALTHY PERSONS

BY LESLIE T. WEBSTER, M.D., AND THOMAS P. HUGHES

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

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Present knowledge of the epidemiology of human pneumococcus infection is incomplete and confused. In the first place, it is uncertain whether the pneumococcus group of bacteria consists of one, two, perhaps three specific organisms, Types I, II, and III, each with an undetermined capacity for variation, or of a relatively large collection of definite entities, each with general similarities but with specific differences in variability, in ability to spread in a population, to grow in human tissues at the normal portal of entry, and to incite the phenomena of disease. A further question then arises as to what is the respective ability of each type or strain of pneumococcus to vary, to spread, and to incite disease. Finally, it may be asked, what are the respective rôles of the microorganism and of the host in determining the various phases of individual infection on the one hand—the healthy carrier state, the localization of infection in the pharynx, sinuses, and middle ear, and the generalization of infection in the lungs, peritoneum, meninges, and blood-stream—and population infection on the other hand—epidemic, sporadic, and endemic prevalence?

Answers to similar questions in the field of animal infections have been obtained by controlled experimentation under natural conditions (1), but in the case of human pneumococcus infection such procedures are not feasible. Consequently, a survey has been made of the incidence of pneumococci in healthy persons in the hope that the resulting findings, although lacking the degree of control necessary for drawing precise conclusions, will be useful in reaching an understanding of (a) the specificity and stability of pneumococci, (b) the different capacities of pneumococci to infect and give rise to disease, and (c)

Observations on the 22 children of Group IV made during May and June, 1930, were as follows: 4 cultures taken from the nasal passages and throat of each individual showed pneumococci in 41 per cent of the group. The organisms were found in nasal cultures of 37 per cent and throat cultures of 23 per cent. Cultures

TABLE I

Incidence of Pneumococci on Nose and Throat Cultures of 105 Normal Persons

Serum type of pneumococcus	Number of individuals positive	Individuals positive per cent	Duration of positive period in different individuals	Mouse virulence of strains from different individuals ¹
I	1	0.9	1± day	6
II	2	1.9	1±, 1± day	5-, 5, 6
III	9	8.5	1, 1, 1, 1, 1, 1, 1 day± 3, 8 mos.	6-, 6-, 6-, 5-, 6-, 5-, 5, 5, 6-, 5, 6
VII	1	0.9	7 mos.	1 cc., 1 cc., 1 cc.
X	2	1.9	1, 1 day±	1
XIII	9	8.5	1, 1 day±; 2+, 4+, 4+, 5, 5 mos.; 1+, 3½+ yrs.	Avir.-, 1-, 2, 1, Avir.-, 3, 4, 3-, 2, 3-, 5, 5, 5-, 3, 3, 4, 3-, 3, 4, 3, 2, 2, 2, 4, 4, 5, 3, 4
XIV	5	4.7	1, 1, 1 day±; 2+, 3+ mos.	1-, 1-, 1-, 1, 0, 1, 2, 2-
XVII	1	0.9	3+ mos.	2, 1, 2, 2
XVIII	3	2.8	1, 1 day±; 7+ mos.	2-, 1 cc.-, 5, 5, 5
XIX	2	1.9	1 day ±; 4+ mos.	1 cc.-, 1, 1, 1, 1, 1
XXIII	1	0.9	1 day±	1 cc.
x ²	9	8.5	4+, 4+, 4+, 5, 5 mos.; 1½+, 2+ yrs.	4, 3, 3-, 1, 2-, 3, 3, 1 cc., 4-, 1, 1, 2, 2, 1, 2, 2, 1 cc., 1, 2, 1-, 2, 2, 2, 1 cc., 1, 2, 1, 1, 1, 1-, 3, 3, 3, 4, 3-, Avir. (14 strains tested)
Group IV ³	51	48.5		
Non-type-specific	13	12.4		

¹ Results of tests on consecutive strains from a given individual are given together between dashes and are expressed exponentially. Avir. = avirulent; 1 cc. = 1 cc. killed in 48 hours; 1 = a dilution of 10⁻¹ killed; etc.

² Strains from each individual agglutinated in a homologous serum and in no other sera.

³ Strains agglutinated in no available sera. No homologous serum prepared.

from the throats of individuals of this group contained pneumococci less frequently than cultures from the nasal passages—6 of 84 cultures from the throat, 7 per cent, as compared to 11 of 84 cultures from the nose, 13 per cent.

numbers of Gram-positive cocci, Gram-negative cocci, Gram-positive bacilli, diphtheroids, Gram-negative bacilli, hemolytic, green-producing, and indifferent streptococci, hemoglobinophilic bacilli, and pneumococci. The procedure for identifying pneumococci was to transfer representative, small, green-producing colonies suggestive of pneumococcus to blood agar plates or slants and after 6 to 8 hours' incubation to reseed this pure culture growth to broth. The broth cultures, incubated 6 to 9 hours, were then tested for solubility in bile, fermentation of inulin, autolysis in saline, agglutination in acid buffers (1 α), agglutination in pneumococcus sera of Types I to XXV,² and virulence for mice. The virulence of the cultures for mice was tested by injecting 1 cc. of the 8 hour broth culture in dilutions of 10^0 to 10^{-6} intraperitoneally into a total of 14 mice, each dilution of culture being given to two mice. The result was expressed in terms of dilution of culture killing all mice within 48 hours.

GENERAL RESULTS

Observations on the 39 individuals of Group I were made for periods averaging 11 months, during which an average of 39 cultures were secured from the nasal passages and 27 from the throats of each individual. Pneumococci were obtained from 86 per cent of the group at some time during the period, from the nasal passages of 35 per cent, and from the throats of 86 per cent. Cultures from the throats of individuals of this group contained pneumococci more frequently than cultures from the nasal passages—249 of 913 cultures from the throat, 27.3 per cent, as compared to 71 of 1,330 cultures from the nose, 5.3 per cent.

Observations on the 19 children of Group II were made from November, 1929, to March, 1930, during which time five cultures from nasal passages and throat were secured from each individual. Pneumococci were obtained from 69 per cent of the group, from the nasal passages of 69 per cent and from the throats of 38 per cent. Cultures from the throats of members of this group contained pneumococci less frequently than cultures from the nasal passages—6 of 101 cultures from the throat, 6 per cent, as compared to 40 of 101 cultures from the nose, 40 per cent.

Observations on the 25 children of Group III were made from February to June, 1930, during which time 4 cultures were taken from the nasal passages and throat of each individual. Pneumococci were obtained from 80 per cent of the group, from the nasal passages of 72 per cent and the throats of 72 per cent. Pneumococci were obtained on cultures from the throat and nasal passages with equal frequency—33 of 111 cultures from the throat, 30 per cent, and 35 of 111 cultures from the nose, 31 per cent.

² Diagnostic sera of Types I, II, and III were kindly furnished by Dr. Mary B. Kirkbride of the New York State Department of Health Laboratories; sera of Types IV to XXV by Dr. G. Cooper of the New York City Department of Health Laboratories.

To recapitulate, pneumococci were found at one time or another in cultures from the nasal passages or throats of about 80 per cent of the 105 healthy adults and children studied. In adults they occurred chiefly on cultures from the throat, while in children, they occurred more frequently on cultures from the nasal passages.

Specificity of Pneumococci Found in Healthy Persons

Of about 500 strains of pneumococci obtained from the four groups of individuals, 97 per cent proved to be serologically specific. They formed smooth colonies, were bile-soluble, were for the most part virulent for mice, did not flocculate in acid buffers, but agglutinated in specific antipneumococcus serum. The incidence of the various type-specific strains among the 105 individuals is given in Table I. Types III, XIII, XIV, and XVIII were obtained most frequently. Sixteen atypical strains from 13 persons were soluble in bile, fermented inulin, failed to kill mice in 1 cc. doses, did not flocculate in normal salt solution, but flocculated over a wide range of acid buffer solutions, pH 3.6 to 4.7 (1 *a*), and agglutinated in all types of antipneumococcus serum employed. The colony morphology of these strains differed markedly; some colonies were disc-shaped, others flat, others pin-head, and still others granular. These cultures are defined in the present paper as non-type-specific strains.

Stability of Pneumococci in Normal Persons

Evidence of the stability of pneumococci growing in the nasal passages and throats of normal people was secured by comparing strains obtained on successive cultures from the same individual. Strains of pneumococci isolated from a single carrier on successive tests proved with rare exceptions to be of the same serological type and were uniform in colony morphology, bile solubility, tendency to autolyze, inulin fermentation, agglutinative properties, and virulence for mice.

Thus, 11 strains from the 3 month carrier of Type III pneumococci were alike (Table I), and 29 strains from the 8 month carrier of Type III pneumococci were alike; moreover, 18 strains from the 7 month carrier of Type VII pneumococci, 14 strains from a 5 month carrier of Type XIII organisms, 17 strains from the 1 year carrier of Type XIII, and 52 strains from the 31 year carrier of Type XIII pneumococci were alike; 18 strains from a 5 month carrier of an unnamed specific

type of pneumococcus, 20 strains from another 5 month carrier of pneumococci of the same type, 32 strains from an 18 month carrier of a different unnamed specific type of pneumococcus, and 51 strains from a 2 year carrier of still another unnamed specific type, were uniform respectively. On rare occasions, these carrier strains were accompanied by small numbers of pneumococci of a different serological type, but there was no evidence suggestive that any carrier strain was undergoing a type-transformation.

This degree of uniformity in successive strains of pneumococci recovered from a given carrier indicates that pneumococci growing in the upper respiratory tract of healthy humans are relatively stable.

A further measure for testing the stability of pneumococci in healthy people was suggested by the observations of Griffith (3) and Dawson and Avery (4) that type-transformation and virulence-enhancement of pneumococci *in vitro* take place not in the type-specific smooth colony forms but in the degraded or rough variants. An attempt was made, therefore, to determine whether the presence of non-type-specific pneumococci obtained in nose and throat cultures of the 13 persons referred to previously (Table I), was associated in any way with type-transformations or virulence changes *in vivo*. In each case non-type-specific pneumococci were secured infrequently and in very small numbers. They were found during periods in which no other pneumococci were present, and during or at the end of periods of 1 month to 3 years in which the same type-specific and stable pneumococcus was present. These relationships are shown in the partial protocols of 4 cases (Table II). There is nothing in these observations to indicate that the occurrence of non-type-specific pneumococci was associated with a type-transformation or virulence-enhancement process *in vivo*.

Relative Capacity of Pneumococci of Different Types to Survive and Spread in Healthy People

It is known that pneumococci of Types I and II are found most frequently in cases of lobar pneumonia and relatively infrequently in healthy persons (5, 6, 7). In the present group of 105 individuals from whom a total of more than 3,000 cultures has been taken, Type II pneumococci were found on but three single cultures from two individuals and Type I was obtained on but one occasion from a single

	1930									
	1929		1930		1930		1930		1931	
	Date of culture		Jan.		Feb.		Mar.		Apr. to May	
31	Dec. to Jan.		21		4		18		17	
	14 19		22		13		27		21	
	4 tests 0		x		x		x		3 tests 0	
35	Date of culture		1928		1928		1929		1930	
	Sept. to Oct.		Oct.		Nov.		Jan.		Jan. to May	
	3 28		28		3		5 6 7 8		21 12	
35	Type of pneu- mococci		GIV		GIV		A 0 0 A		12 tests x	
	17 tests 0		GIV		17 tests 0		50 tests 0		45 tests 0	
	Date of culture		Sept. to Oct.		Nov. to Jan.		Jan. to Jan.		June to Jan.	
35	3 28		28		4 4		9 11		13 31	
	17 tests 0		GIV		17 tests 0		50 tests 0		45 tests 0	
	Type of pneu- mococci		GIV		GIV		A 0 0 A		12 tests x	

A = Atypical pneumococcus.

GIV = Strains agglutinated in no available sera. No homologous sera prepared.

x = All strains from the individual agglutinated in homologous serum but in no other available sera.

0 = Pneumococci absent.

they were actually spreading from individual to individual (Tables III and IV).

Of the nine persons yielding Type III pneumococci, seven were working in three adjacent rooms (Table III). Of five persons tested in one of these rooms, two were positive, one for 3 months and one throughout an 8 months' period of observation. Of seven persons tested in an adjacent room, three were positive on four, one, and one isolated occasions respectively; and of five persons tested in another adjacent room, two were positive on one occasion. It is important to note again that these strains of Type III pneumococci killed mice uniformly within 48 hours when injected intraperitoneally in dilutions of 10^{-5} and 10^{-6} (Table I).

Of the nine persons positive for Type XIII pneumococci, six were working whole or part time in a single laboratory room and one in an adjacent room (Table IV). Case 15 has been a carrier for $3\frac{1}{2}$ years; her husband is likewise a carrier of Type XIII. Case 22, working within a few feet of Case 15, became a carrier of Type XIII in January, 1929, and remained so until April, 1930. Cases 36, 20, and 52, working in the same room, have each been positive on one occasion; Case 33, a part time assistant, and No. 10, working in an adjacent laboratory, became carriers of Type XIII in the autumn of 1929 and remained so until the spring of 1930.

Apparently the types of pneumococci which are more commonly associated with cases of serious disease (Types I and II) survive and spread with relative infrequency in normal persons, and conversely, the types of pneumococci less commonly associated with cases of severe disease (Types III, XIII, etc.) are those which tend to survive and spread in these individuals.

Thus far it has been shown that 97 per cent of pneumococci obtained from about 3,000 cultures from the nasal passages and throats of 105 healthy persons were serologically specific, that these specific strains, especially those obtained from chronic carriers which could be studied more carefully, proved to be stable in colony morphology, bile-solubility, tendency to autolyze, acid and serum agglutination characteristics, and intraperitoneal virulence for mice. Atypical strains, on the other hand, found in a few individuals on single and scattered occasions, appeared not to be undergoing type-transformation into Types I, II, or other serologically specific strains. Finally, Types I and II pneumococci, rarely encountered in the present group of healthy persons, showed little tendency to survive or spread, while Types III and XIII, present in 10 per cent of the group, showed a decided tendency both to survive and spread.

TABLE III

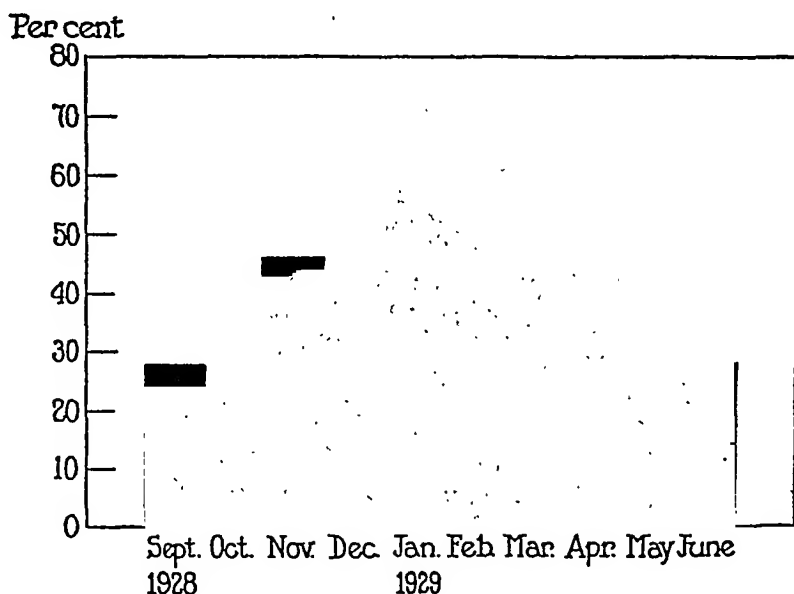
Room number	Number persons tested	Number persons positive	Identity of positive cases	Duration of carrier period							
				1928		1929					
				Oct.	Dec.	Feb.	Apr.	June	Aug.	Oct.	Dec.
1	5	2	12 23								
2	7	3	7 30 6								
3	5	2	4 5								

Incidence of Type XIII Pneumococci in Normal Persons

[illegible]

and all throat cultures were negative. In contrast with these findings are the observations that Type III pneumococci and others included in Group IV occur frequently in normal people (5, 6, 7). Types III and XIII, for example, were each found in nine of the present group of 105 individuals (Table I), and under circumstances suggestive that

These differences in the incidence of pneumococci in individuals have been too characteristic and consistent to be attributable to chance and too apparent in members of the same family and in individuals working in the same and adjacent rooms to be related to qualitative or quantitative differences in exposure to pneumococci. In the case of 17 persons exposed to a carrier of Type III pneumococcus (page 544), eleven remained consistently negative, four were positive on single isolated occasions, and one became a carrier for 3 months (Table III); in the case of seven individuals exposed to a carrier of



TEXT-FIG. 1. Incidence of pneumococci in cultures from the nose and throat of individuals of Group 1, 1928-1929.

Type XIII pneumococcus (page 544), one remained consistently pneumococcus-free, two were positive on single occasions, and three became Type XIII carriers for 7 and 15 months respectively (Table IV).

Apparently these differences in the incidence of pneumococci were associated with host properties. There is reason to believe that the pneumococcus carriers were infected individuals in the sense that the organisms were growing in the rhinopharyngeal tissues. Five of the eight chronic carriers had sinus or tonsil infections and suffered four

Individual Differences in the Incidence of Pneumococci on Nose and Throat Cultures

During the course of these studies it became apparent that there were consistent differences in the bacterial flora of the nasal passages and throats of different individuals. With respect to the presence or absence of pneumococci on these cultures, the 39 individuals of Group I were divided into non-carriers, transient, periodic, and chronic carriers (Table V). *Non-carriers* comprised eleven of the group, of which five were negative whenever tested and six were positive on only one or two occasions. *Transient carriers*, twelve in number, were positive for pneumococci on single and scattered occasions between

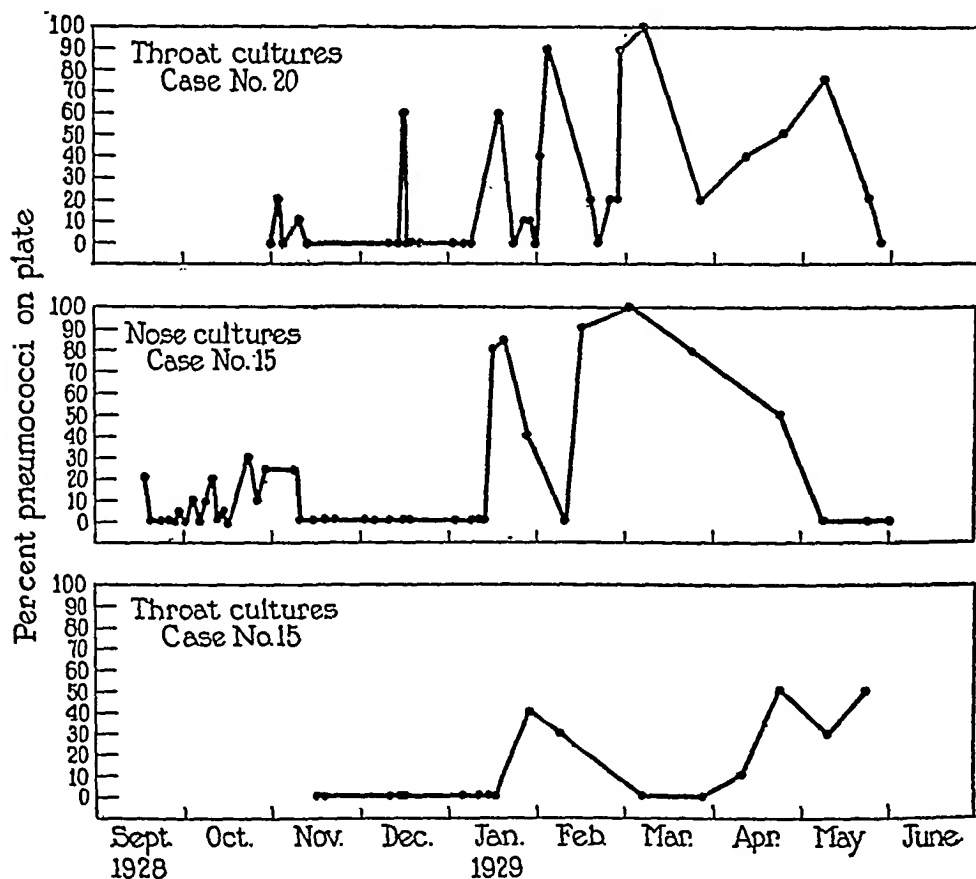
TABLE V

Individual Differences in the Incidence of Pneumococcus on Nose and Throat Cultures

Class	Description	Number	Per cent
Non-carriers	Pneumococcus-free	5	13
	Positive 1-2 tests	6	15
Transient carriers	Positive tests; occasional and scattered	12	31
Periodic carriers	Positive tests; 1 week to 3 months	8	21
Chronic carriers	Positive tests; 3 months to 3+ years	8	21

pneumococcus-free periods (Table II, Cases 5 and 22). The strains of pneumococci recovered from an individual of this group on an occasion following a pneumococcus-free period, usually differed from the strains obtained on a subsequent occasion following another pneumococcus-free period. *Periodic carriers*, numbering eight, were those from whom pneumococci of one serological type were obtained for periods of 1 to 12 weeks between pneumococcus-free intervals (Table II, Case 35). *Chronic carriers*, numbering eight, were those from whom pneumococci of one serological type were obtained for periods of 3 months to 3 years or more (Table II, Case 20).

previously and subsequently were pneumococcus-free. None of the five non-carriers of pneumococci, on the other hand, had either objective or subjective evidence of upper respiratory tract infection, nor contracted coryza or sore throat during the 7 month to 3 year periods of observation. Finally, it was noted that the incidence of pneumococci in individuals of Group I underwent a seasonal variation (Text-



TEXT-FIG. 2. Incidence of pneumococci in cultures from the nose and throat of Cases 20 and 15, 1928-1929.

fig. 1) similar to that of the incidence of coryza and sore throat in the same individuals (Table VI) and that the incidence of the given type of pneumococcus in each of the chronic carriers was subject to the same sort of variation (Text-fig. 2). These observations are suggestive that the persons studied differed in the amount of their resistance to pneumococcus infection and that this resistance underwent a seasonal fluctuation.

or more attacks of coryza, sore throat, headache, or malaise during each winter. During these attacks the pneumococci on throat cul-

TABLE VI

Incidence and Duration of Coryza and Sore Throat in 30 Persons of Group I, 1928-1929

Identity of case	1928				1929					
	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June
1			—	—		—				
2		—	—							
3				—	—		—			
4										
5										
6			—		—			—		
7										
8							—			
9	—			—	—					
10			—				—			
12		—					—			
13										
14										
15			—		—					
16					—	—				
17										
18				—	—					
19					—					
20		—	—	—	—	—				
22				—		—				
23				—	—	—				
25		—			—					
26				—						
28				—		—				
29		—	—							
30				—						
31					—					
32					—	—	—			
35				—	—	—				
36			—		—					
Totals...	0	6	8	11	14	8	5	1	0	0

ture plates increased in numbers markedly, often to the point of being present in pure culture, and were found on nasal culture plates which

results of experimental epidemiological studies on analogous animal infections. With reference to the question of specificity and stability of the bacterial incitant, it was found that in rabbit and fowl pasteurellosis specific strains of *pasteurella* from cases of septicemia, pneumonia, sinusitis, and from healthy carriers were on all significant occasions under natural and controlled conditions, relatively fixed in pathogenicity and serological specificity (1 *b, c*). With reference to the question of differences in the capacities of strains to survive, spread from host to host, and to give rise to disease, it was determined experimentally that strains of *pasteurella* most able to incite severe disease were least able to spread from host to host, and *vice versa* (1 *d, e*). Finally, regarding the problem of respective rôles of microorganism and host in determining the spread of individual and population infection studies on three populations of rabbits infected with *pasteurella* under relatively controlled conditions showed that some animals were consistently free of the organisms, others were periodic or chronic carriers, and still others carried *pasteurella* in association with sinusitis or pneumonia (1 *f, g, h*). Moreover, controlled groups of rabbits given a fixed intranasal dose of *pasteurella* reacted similarly according to the above classification (1 *d*), and similar tests with fowls and *P. avicida* gave corresponding results (1 *e*). Finally, similar differences in the response of individual animals to pathogenic microorganisms were shown to depend upon differences in the resistance of the animals (1 *i*). From these studies, it has been concluded that the prevalence of these infections is determined by variations and differences in the resistance of individual animals to the relatively stable bacterial incitant.

The similarity in behavior of these animal infections and human pneumococcus infection is suggestive of the possibility that their incidence and spread are controlled by similar mechanisms, that in the case of pneumococcus infection, the pneumococcus incitants differ in their ability to spread from host to host and grow in tissues at the portal of entry, and to incite severe disease, but that each is relatively unchanging, that the epidemic and endemic spread of infection is determined by differences and variations in host resistance to the given strains of organisms. For the solution of the problem, however, evidence is required which is based on a satisfactory experimental technique involving controlled and natural conditions.

DISCUSSION

The bearing of the foregoing data on the question of pneumococcus epidemiology is threefold. In the first place, most pneumococci in healthy persons proved to be antigenically specific and stable, an observation in agreement with the knowledge that in persons with pneumonia, pneumococci are specific (5 to 11) and stable. Pneumococci kept under highly artificial conditions, however, are reported to undergo profound changes in serological specificity and virulence for mice (3, 4). One is inclined to believe, therefore, that the group of bacteria classified as pneumococci consists of a collection of biological entities with different specific characteristics which, although varying in abnormal environments, are in man relatively unchanging (12). Second, pneumococci of Types I and II were obtained on but three occasions, while pneumococci of Types III and XIII were encountered frequently and under conditions suggestive that they were actually spreading from person to person. These findings are in agreement with the knowledge that pneumococci of Types I and II are relatively common in cases of severe pneumonia and uncommon in healthy persons (6 to 8), and together are supportive of the view that pneumococci differ among themselves in their inherent capacity to spread from host to host and multiply in tissues at the normal portal of entry and in their capacity to incite natural disease. Third, individuals appeared to differ in their response to exposure to pneumococci, some remained consistently pneumococcus-free, some were transient carriers, others, periodic carriers, and still others, chronic carriers. Moreover, the number of pneumococcus carriers and the quantity of pneumococci obtained from each chronic carrier underwent a seasonal variation corresponding to that of the incidence of coryza, sore throat, and other upper respiratory tract disease. The observations are suggestive that pneumococcus carriers are infected individuals in the sense that the organisms are growing in their nasopharyngeal tissues, that the presence or absence of the infected state is dependent on the amount of resistance of the host to the given pneumococcus, and that this resistance is a varying property, decreasing during the winter months and increasing during the warm weather.

These observations may be considered to advantage in the light of

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SUMMARY

1. Pneumococci were obtained at one time or another from the nasal passages or throats of 80 per cent of 105 adults and children studied. In adults, they were obtained more frequently from the throat; in children, as often from the nasal passages as from the throat.

2. Of 500 pneumococcus strains studied, 97 per cent proved to be serologically specific. They formed smooth colonies and were for the most part avirulent for mice. Types I and II were obtained from one and two individuals respectively on one occasion only. Type III was obtained from nine individuals; Type XIII from nine individuals; Type XVI and Type XVIII from three individuals, for varying periods in each case. Atypical pneumococci were secured from 13 persons on single and scattered occasions. They varied in colony morphology, did not kill mice, or agglutinate in saline, but flocculated in all types of antipneumococcus sera employed and over a wide pH range in acid buffers. Their occurrence was apparently not associated with any type-transformation or virulence-enhancement process *in vivo*.

3. Strains of pneumococcus obtained on successive cultures from a given carrier were, with rare exceptions, of the same serological type and were similar in colony morphology, virulence for mice, and other tested biological characteristics.

4. Pneumococci of Types I and II were obtained under conditions suggestive that they lacked a capacity to spread readily; pneumococci of Types III and XIII, on the other hand, were obtained under conditions suggestive that they were spreading from person to person.

5. The persons studied differed consistently with respect to the occurrence of pneumococci. Some were pneumococcus-free, some were transient carriers, some periodic, and some chronic carriers. Data are given which suggest that the differences were due to variations in host resistance.

6. The incidence of pneumococci in all individuals studied underwent a seasonal variation paralleling that of coryza and sore throats in the same persons.

only occasionally and then only in a ratio of 1 part of virus emulsion to 3 parts of serum. These workers also attempted without success to produce an antiserum from sheep. At the same time they also tested Rosenow's antistreptococcus serum of alleged therapeutic value in poliomyelitis and found it to be devoid of any virucidal principles.

Herein is reported the successful production of potent virus-neutralizing preparations drawn from immunized horses with evidence relative to its potency from the standpoint of prophylaxis and therapeutics.

Method

In general it has been found satisfactory to inject horses with virulent cord emulsion (10 per cent) in doses increasing from 1 cc. to 50 cc. The inoculations are given subcutaneously or intradermally and are administered on 4 successive days in each week. That certain horses fail to respond has been demonstrated. It has also been found that horses having no measurable native antibody may readily become producers of a high titre serum. Indeed, in no horse has there yet been found evidence of a specific antibody prior to the immunization treatments.

After 2 months of injections, horses which will satisfactorily respond to the immunization will have produced sufficient antibody to neutralize the active virus of poliomyelitis. Evidence of worth is determined by means of an *in vitro* neutralization test, the technique of which is as follows:

5 per cent virus emulsions are prepared by grinding virulent monkey spinal cord with saline. The serums to be tested are added in the desired proportion and after an hour's incubation at room temperature the mixtures are introduced into the cerebral hemispheres of susceptible monkeys. The amount of inoculum is so calculated that it represents at least two infective doses. The potency of the virus has apparently no effect upon the amount of a specified serum preparation needed to neutralize it. When using particularly potent strains of virus the inoculum often contains as many as several hundred killing doses of virus.

Using this procedure it has been possible to determine several relative values quite accurately. For example, pooled convalescent serum from human poliomyelitis cases will consistently neutralize 5 per cent virus emulsion in the ratio of 1 part of serum to 20 parts of virus but fails to do so when the ratio is 1:30. This has been demonstrated by the titration of two pools of convalescent serum each containing serum from sixteen individuals.

Individual serums from so-called normal human adults will often

ORTHOPATHIC HOSPITAL L

A POTENT ANTIPOLIOMYELITIC HORSE SERUM CONCENTRATE AND ITS EXPERIMENTAL USE IN INFECTED MONKEYS*

By ELLIOTT R. WEYER, PH.D., WILLIAM H. PARK, M.D., AND
E. J. BANZHAF, PH.D.

(From the Laboratories of New York University and the Department of Health of the City of New York, New York)

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Since the discovery by Netter (1) and Levaditi (2) of the presence of virus-neutralizing antibodies in the blood, both from human convalescents and from monkeys recovered from experimental poliomyelitis, several attempts have been made to immunize large animals with a view to quantity production of therapeutic serum.

The results of Aycock (3) and others who have used convalescent serum in the treatment of poliomyelitis tend to indicate that serum therapy is effective when used in the preparalytic stage of this disease. The difficulties encountered in obtaining ample quantities of human immune serum have hampered its widespread use.

Flexner (4), in 1910, reported unsuccessful attempts to bring forth an immunological response in a horse by injection over a period of many months of filtrates carrying the active virus of poliomyelitis.

In 1917, Banzhaf and Neustaedter (5) published the results of similar efforts, having injected subcutaneously and intramuscularly the supernatant fluid from emulsions of brains and spinal cords of monkeys dying of poliomyelitis. This antiserum gave some evidence of ability to neutralize virus in the few experiments then conducted.

Pettit (6), in 1918 described the preparation and subsequent use in human cases of a horse serum drawn from an animal which had received injections over a period of 2 years. He did not test this serum directly for virus-neutralizing antibodies but depended upon clinical results for an index of its usefulness. Later, Stewart and Haselbauer (7) investigated this preparation and found it to neutralize virus

*The expenses of this experimental study were largely defrayed by a gift of money to New York University by Mr. Jeremiah Milbank, this University being one of the group working under the International Committee for the Study of Infantile Paralysis.

TABLE II
Progress of Horse 4

Monkey No.	Mixture inoculated	Virus No.	Serum tested	Ratio virus to serum	Paralysis appeared	Death	Result
	cc.				days	days	
250	0.4	145	Poliomyelitis, Horse 4, initial bleeding, 9/13/27	5:1	7	7	Poliomyelitis
30	0.4	145		10:1	7	8	"
79	0.4	145		20:1	7	8	"
214	0.4	145	Poliomyelitis, Horse 4, bleeding of 1/14/29	10:1	—	—	Remained well
34	0.4	145		20:1	9	10	Poliomyelitis
253	0.4	145		20:1	20	23	"
254	0.4	145		25:1	14	16	"
228	0.4	145	Control	—	7	9	"
259	0.3	145	"	—	4	4	"
268	0.4	248	Poliomyelitis, Horse 4, bleeding of 2/21/29	20:1	9	10	"
269	0.4	248		25:1	7	8	"
252	0.3	248	Control	—	7	10	"
261	0.15	248	"	—	9	10	"
314	0.4	298	Poliomyelitis, Horse 4, bleeding of 3/20/29	20:1	—	—	Remained well
315	0.4	298		10:1	—	—	" "
316	0.4	298	Poliomyelitis, Horse 4, bleeding of 5/8/29	20:1	17	21	Poliomyelitis
317	0.4	298		10:1	—	—	Remained well
311	0.3	298	Control	—	6	14	Poliomyelitis
312	0.15	298	"	—	6	14	"
285	0.4	284	Poliomyelitis, Horse 4, bleeding of 7/5/29	66:1	9	10	"
329	0.4	285		50:1	13	15	"
328	0.4	320		20:1	—	—	Remained well
315	0.4	320	Control	10:1	—	—	" "
314	0.3	320		—	7	13	Poliomyelitis
317	0.15	320	"	—	6	19	"
327	0.3	284	"	—	6	6	"
313	0.15	284	"	—	6	6	"
330	0.4	285	Poliomyelitis, Horse 4, bleeding of 8/26/29	25:1	—	—	Remained well
335	0.3	285	Control	—	8	11	Poliomyelitis
336	0.15	285	"	—	7	Killed	"

TABLE I
Virucidal Properties of Various Human Sera

Monkey No.	Mixture injected	Virus used	Serum tested	Ratio virus to serum	Paralysis appeared	Death	Result
	cc.				days	days	
Human convalescent serum pooled							
228	0.3	226	A	10:1	—	—	Not infected
220	0.3	226	"	20:1	—	—	" "
227	0.3	226	"	40:1	7	9	Poliomyelitis
214	0.3	89	B	20:1	—	—	Not infected
"Normal" adult serum							
294	0.4	267	Donor 1	10:1	—	—	Not infected
293	0.4	267	" 1	20:1	8	9	Poliomyelitis
310	0.4	298	" 2	14:1	—	—	Not infected
309	0.4	298	" 3	14:1	10	15	Poliomyelitis
305	0.4	298	" 4	14:1	6	14	"
462	0.4	457	" 5	10:1	8	10	"
466	0.8	10	" 5	1:1	—	—	Not infected
384	0.4	457	" 6	10:1	9	12	Poliomyelitis
467	0.8	10	" 6	1:1	—	—	Not infected
385	0.4	457	" 7	10:1	—	—	" "
458	0.4	457	" 8	10:1	19	—	" "
468	0.4	10	" 9	10:1	10	11	Infected, killed
311	0.3	298	Control, no serum	—	6	14	Poliomyelitis
312	0.15	298	" " "	—	6	14	"
279	0.3	267	" " "	—	6	7	"
282	0.15	267	" " "	—	7	9	"
475	0.3	10	" " "	—	8	11	Infected, killed
458	0.15	10	" " "	—	8	—	Lived, paralyzed
460	0.3	457	" " "	—	11	—	" "
461	0.15	457	" " "	—	11	—	" "
210	0.5	89	" " "	—	8	9	Poliomyelitis
231	0.3	226	" " "	—	8	9	"
252	0.3	248	" " "	—	7	10	"
261	0.15	248	" " "	—	9	10	"
215	0.5	89	Virus emulsion + saline + tricresol	10:1	9	11	"

effect a neutralization in a ratio of 1:10, but usually fail in 1:15. According to our observations four out of nine were effective in 1:10.

This knowledge is of practical value in that it is not necessary to search for convalescent cases for a supply of effective serum.

16 months with virus of very dubious potency. Bleedings, during and subsequent to this treatment failed to show any specific antibody.

The inoculations with emulsions of known virulence, were resumed in October, 1928, this time intravenously. This work was begun on a more elaborate scale because of the fund given by Mr. Jeremiah

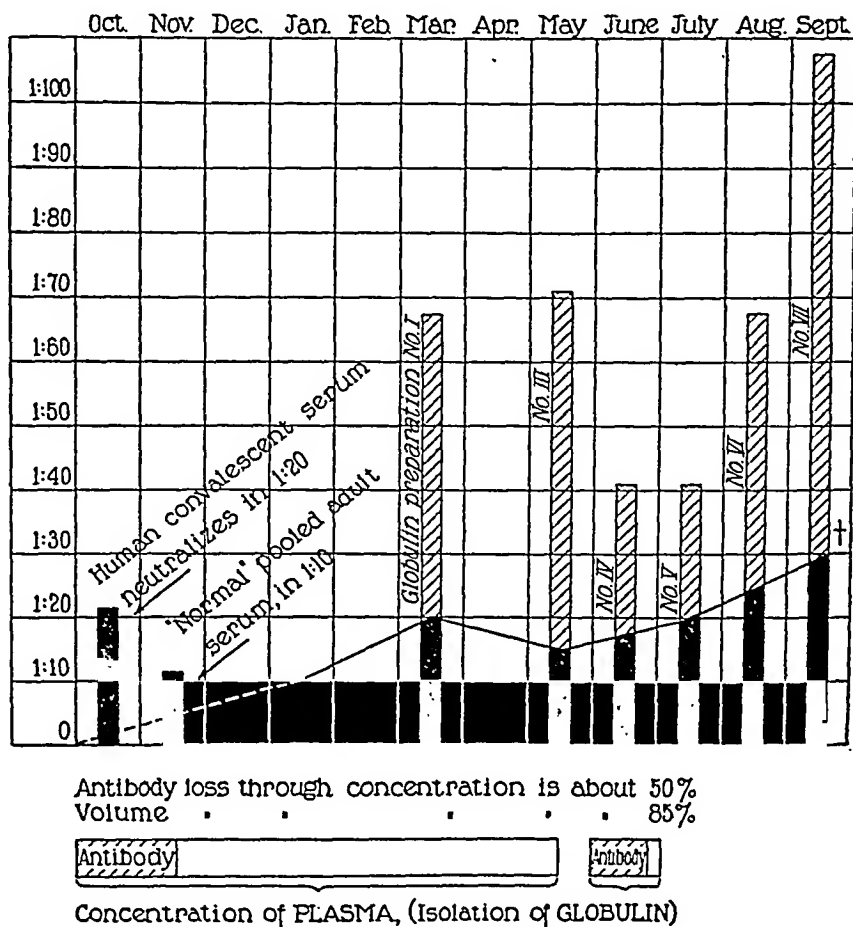


CHART 1

Milbank. Encouraging results were obtained and in the following January the serum was capable of neutralization in the ratio of 10:1. The titre continued to mount and bulk bleedings were made from time to time when late in the summer the animal died from an obscure infection which resulted in the death of several other horses in the same stable. A preliminary report dealing with the successful immunization

TABLE III

Serum Preparations from Horse 4 Refined by Banzhaf

Monkey No.	Mixture inoculated	Virus No.	Serum tested	Ratio virus to serum	Paralysis appeared		Result
					Days	Days	
275	0.4	252	Globulin preparation 1	100:1	5	6	Poliomyelitis
276	0.4	252	" " 1	66:1	—	—	Remained well
313	0.4	298	" " 3	40:1	—	—	" "
308	0.4	320	" " 3	50:1	10	13	Poliomyelitis
321	0.4	320	" " 3	70:1	—	—	Remained well
322	0.5	320	" " 4	40:1	—	—	" "
323	0.5	320	" " 5	40:1	—	—	" "
345	0.4	285	" " 5	25:1	—	—	" "
330	0.4	285	" " 5	50:1	21	26	Poliomyelitis
357	0.4	285	" " 5	66:1	12	14	"
336	0.4	285	" " 6	66:1	—	—	Remained well
334	0.4	285	" " 6	100:1	8	11	Poliomyelitis
365	0.4	402	" " 6	66:1	—	—	Remained well
398	0.4	402	" " 6	100:1	—	—	" "
367	0.4	402	" " 7	66:1	—	—	" "
368	0.4	402	" " 7	100:1	—	—	" "
444	0.4	9	" " 7	125:1	17	20	Poliomyelitis
443	0.4	9	" " 7	156:1	11	14	"
277	0.4	252	Control	—	5	8	"
311	0.3	298	"	—	6	14	"
312	0.15	298	"	—	6	14	"
314	0.3	320	"	—	7	13	"
317	0.15	320	"	—	6	19	"
354	0.3	285	"	—	7	7	"
355	0.3	285	"	—	9	10	"
356	0.15	285	"	—	9	14	"
417	0.3	402	"	—	7	7	"
418	0.1	402	"	—	10	10	"
376	0.6	402	Normal horse serum Control A	1:1	9	9	"
377	0.6	402	" " " " B	1:1	9	10	"
385	0.6	402	" " " " C	1:1	10	12	"
442	0.6	9	" " " " D	1:1	7	12	"

The Results of Immunization

Referring now to the horses, the first animal, Horse 4, had previously been injected subcutaneously by one of us (Banzhaf) over a period of

TABLE IV

Protocols of Horses 5 and 6, Failure to Respond

Monkey No.	Mixture inoculated	Virus No.	Serum tested	Ratio virus to serum	Paralysis appeared	Death	Result
	cc.				days	days	
<u>4/2/29</u>							
265	0.4	277	Horse 5, initial bleeding	5:1	7	10	Poliomyelitis
263	0.4	277	" 6, " "	5:1	7	10	"
<u>5/13/29</u>							
281	0.4	267	" 5, bled 5/7	20:1	7	8	"
286	0.4	267	" 5, " 5/7	10:1	7	8	"
288	0.4	267	" 6, " 5/7	20:1	5	7	"
289	0.4	267	" 6, " 5/7	10:1	9	10	"
<u>6/27/29</u>							
318	0.4	298	" 5, " 6/24	5:1	10	17	"
319	0.4	298	" 6, " 6/24	5:1	11	18	"
320	0.3	298	Control	—	8	11	"
<u>7/17/29</u>							
324	0.4	320	Horse 5, bled 7/15	5:1	7	9	"
325	0.4	320	" 6, " 7/15	5:1	12	17	"
314	0.3	320	Control	—	7	13	"
317	0.15	320	"	—	6	19	"
<u>8/21/29</u>							
323	0.4	314	Horse 5, bled 8/21	2.5:1	8	11	"
326	0.4	314	" 6, " 8/21	2.5:1	8	11	"
310	0.15	314	Control	—	8	11	"
<u>9/9/29</u>							
321	0.6	267	Horse 5, bled 9/9/29	1:1	7	10	"
315	0.6	267	" 6, " 9/9/29	1:1	6	10	"
327	0.3	267	Control	—	6	6	"
313	0.15	267	"	—	6	6	"
<u>11/11/29</u>							
364	0.6	285	Horse 5, + Horse 6, bled 11/13/30	1:1	8	9	"
366	0.3	285	Control	—	6	6	"
<u>2/11/30</u>							
377	0.3	402	Horse 5, bled 2/11/30	1:1	9	9	"
376	0.3	402	" 6, " 2/11/30	1:1	9	10	"
417	0.3	402	Control	—	7	7	"
418	0.1	402	"	—	7	10	"
<u>5/20/30</u>							
368	0.5	445	Horse 5, bled 5/20/30	2.5:1	7	11	"

of this horse was presented by Park before the American Association of Pathologists and Bacteriologists at Chicago, March 28th, 1929 (8, 9).

The larger bleedings from this horse were drawn into citrate solution and concentrations were made according to the following scheme:

The method of fractional concentration of antisera with ammonium sulfate (Banzhaf (9)), without the heating process, is followed. We have not up to the present time determined the heat resisting point of the neutralizing antisubstances. After the method mentioned has been carried through to dialyzing free from salts, it will be noted that some precipitation has formed which contains some of the anti-substances. A gentle rotation of the concentrated fluid in the dialyzing bags will resuspend the precipitate. The fluid is measured and three volumes of distilled water added. Sufficient sodium chloride is added to bring the whole to one-twentieth normal (50 cc. normal sodium chloride per litre) and adjusted to pH 5.0-5.1 and placed into a cold room 5-10°C. overnight. The resulting precipitate consists of further inert substances and practically all the chill producing substances usually present in all sera. These substances are removed by filtration through paper pulp. The clear filtrate adjusted to pH 6.8 and allowed to stand overnight for a further inert fibrin like precipitate which may form, if present it is removed by filtration. To the clear filtrate a half volume of saturated ammonium sulfate solution is added to reprecipitate the globulins and antisubstances. The precipitate is recovered by filtration, pressed free from fluid and dialyzed free from salts. To the dialyzed concentrated fluid 1 per cent sodium chloride and 0.5 per cent phenol are added, it is clarified by filtering through paper pulp and passed through a Berkefeld filter to sterilize.

Referring to Chart 1, it will be seen that the increase in globulin concentration from this procedure is three to four-fold and that the preparation of maximum potency is approximately five times as potent as average human convalescent serum.

Following the loss of Horse 4, immunization was commenced upon two other horses, Nos. 5 and 6. The jugular vein was selected as the route of injection in view of the success obtained by that method in Horse 4. Neither horse had any demonstrable "native" antibody to begin with, nor was any acquired though they received the total substance of approximately 25 virulent spinal cords and brains over a period of a year. During this period the route of inoculation was varied and the sequence of injection changed but with the appearance of no neutralizing value, even in a ratio of 1:1, these horses were abandoned in May, 1930.

muscularly and intravenously in each animal. The third horse failed to respond satisfactorily and was discarded. Concentrated preparations from the two producing animals showed complete neutralization in 100:1 or better.

That the mechanism of virus neutralization by the horse serums might be explained by some non-specific phenomenon has been advanced. It is true that the immunization treatment brings forth a decided response with respect to the nerve tissue necessarily contained in the inoculum. This is readily demonstrated by complement fixation or simple flocculation procedures. However, it is hardly conceivable that this flocculation of emulsified cord by the antiserum could inactivate the virus by entrainment or otherwise in view of proof that serum preparations are still potent after removal therefrom of the antinerve tissue bodies by absorption. A monkey was intracranially injected with an incubated mixture composed of virulent cord emulsion and a serum preparation which had previously been incubated for 2 hours with a heavy (20 per cent) emulsion of normal monkey cord and subsequent centrifugalization. Furthermore, horses which have made no anti-virus response have acquired anticord properties.

Pettit (6) and others have advocated that in the absence of suitable antipoliomyelitis serum other serums or indeed any protein substance, *e.g.* milk, may be used intraspinally with amelioration of symptoms effected by the non-specific protein shock resulting therefrom. Our results gleaned from the treatment of infected monkeys tend to indicate that the chance for recovery is in direct ratio to the virucidal potency of the serum used. In addition, the results obtained when the serum preparations were used intraspinally were far better than similar treatment introduced through any other route, even though the amount of serum injected were 3 to 5 times as much. If the spinal cord is chosen as the most advantageous route for treatment, it follows that concentration of antibodies is of prime importance in view of the limited capacity of the canal. (1 to 2 cc. in small monkeys and 5 to 20 cc. in children.)

Having acquired a particularly potent strain of monkey virus which proved sufficiently invasive to produce typical poliomyelitis when the emulsion was dropped into the noses of the monkeys on 3 successive days, experiments represented in the following charts were conducted,

Two more horses were started, June, 1930. The injections were given intradermally in one case and subcutaneously in the other. As will be seen from the protocols, antibody content of the serums rose from an apparent zero value to the vicinity of 25.1 in 5 months in each case.

TABLE V

Monkey No.	Mixture inoculated	Virus No.	Serum tested	Ratio virus to serum	Panlysis appeared	Death	Result
					days	days	
383	0.5	402	Horse 7, serum before treatment	1:1	10	13	Poliomyelitis
417	0.3	402	Control	—	7	7	"
418	0.1	402	"	—	7	10	"
391	0.5	445	Horse 7, after 7 wks.	2.5:1	—	—	No infection
454	0.15	445	Control	—	6	6	Poliomyelitis
452	0.05	445	"	—	7	11	"
448	0.4	9	Horse 7, after 11 wks.	5:1	—	—	No infection
375	0.4	9	" 7, " 11 "	10:1	—	—	" "
447	0.3	9	Control	—	10	12	Poliomyelitis
455	0.15	9	"	—	10	12	"
457	0.5	9	Horse 7, after 17 wks.	20:1	—	—	No infection
463	0.15	9	Control	—	11	14	Poliomyelitis
469	0.4	457	Horse 7, after 25 wks.	30:1	9	11	"
460	0.3	457	Control	—	11	40	"
461	0.15	457	"	—	11	23	"
442	0.4	9	Horse 8, before treatment	1:1	7	12	"
447	0.3	9	Control	—	10	12	"
455	0.15	9	"	—	10	12	"
456	0.5	9	Horse 8, bled after 9 wks.	5:1	—	—	No infection
458	0.5	9	" 8, " " 9 "	20:1	—	—	" "
463	0.15	9	Control	—	11	14	Poliomyelitis
470	0.4	10	Horse 8, after 9 wks.	30:1	9	17	"
475	0.3	10	Control	—	8	11	"
458	0.15	10	"	—	8	40	"

In the case of these two animals, over a portion of the immunizing period, 0.1 per cent of alum was incorporated in the saline used in preparing the emulsions.

Again, in May, 1930, three additional horses were put on the immunizing treatment. By August, two of these horses were found to have responded. The injections were given subcutaneously, intra-

8 controls

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
	⊕	⊕	⊕	⊕					1	4	2	1		-	All died	-		

Human convalescent serum, intraspinally (S), about 1.0 cc

No.																		
394	⊕	⊕	⊕	⊕		S			Paral.					Dying II				
395	⊕	⊕	⊕	⊕				S	Paral.					Dying II				
409	⊕	⊕	⊕	⊕		S							Paral.			Died		
412	⊕	⊕	⊕	⊕				S								Survived		

Horse globulin, intravenously (V), 5.0 cc

	⊕V	⊕	⊕	⊕	⊕													
377	⊕V	⊕	⊕	⊕	⊕											Survived		
380	⊕	⊕	⊕	⊕	V		V						Paral.			Died		
383	⊕	⊕	⊕	⊕			V									Survived		
413	⊕	⊕	⊕	⊕		V										Survived		
407	⊕	⊕	⊕	⊕			V		Paral.				Died					
411	⊕	⊕	⊕	⊕			V									Survived		

Horse globulin, intraspinally (S), about 1.0 cc

	⊕S	⊕	⊕	⊕	⊕													
382	⊕	⊕	⊕	⊕S		S										Survived		
375	⊕	⊕	⊕	⊕		S										Survived		
406	⊕	⊕	⊕	⊕		S										Survived		
376	⊕	⊕	⊕	⊕			S									Survived		
404	⊕	⊕	⊕	⊕			S									Survived		
403	⊕	⊕	⊕	⊕				S								Survived		
430	⊕	⊕	⊕	⊕				S								Survived		
428	⊕	⊕	⊕	⊕					S							Survived		
432	⊕	⊕	⊕	⊕						S				Paral.		Died		

with a view to the determination of the relative merits of human convalescent serum and the concentrated horse product when used intravenously and intraspinally. The virus is doubtless transmitted by way of the nasal mucosa in human contacts so these results should prove of some value from the standpoint of human therapeutics. So

TABLE VI

Monkey No.	Mixture inoculated	Virus No.	Serum tested	Ratio virus to serum	Paralysis appeared	Death	Result
	cc.				days	days	
448	0.5	445	Horse 9, after 12 wks.	2.5:1	—	—	No infection
440	0.4	9	" 9, " 12 "	5:1	—	—	" "
441	0.4	9	" 9, " 12 "	10:1	—	—	" "
454	0.15	445	Control	—	6	6	Poliomyelitis
452	0.05	445	"	—	7	11	"
448	0.4	9	Horse 9, after 20 wks.	20:1	10	20	"
375	0.3	9	Control	—	10	11	" —killed
413	0.1	9	"	—	12	15	" "
471	0.4	10	Horse 9, after 32 wks.	20:1	—	—	No infection
474	0.4	487	" 9, " 32 "	25:1	—	—	" "
475	0.3	10	Control	—	8	11	Poliomyelitis
458	0.15	10	"	—	8	40	"
494	0.3	487	"	—	7	7	"
495	0.15	487	"	—	9	9	"
451	0.5	445	Horse 10, after 10 wks.	2.5:1	7	11	"
454	0.15	445	Control	—	6	6	"
452	0.05	445	"	—	7	11	"
439	0.5	9	Horse 10, after 18 wks.	2.5:1	—	—	No infection
439	0.4	9	" 10, " 18 "	10:1	8	14	Poliomyelitis
463	0.15	9	Control	—	11	14	"
463	0.15	9	"	—	11	14	"
413	0.1	9	"	—	12	15	"

far, we have not found curative measures to be effective when the monkey is inoculated intracranially.

These data were presented by Weyer in a preliminary report read before the American Association of Immunologists on April 16th, 1930. and confirmation of the results with our serum concentrates has been more recently presented by Rhoads (10).

The results from these experiments cause us to consider at once the possibility of utilizing an antipoliomyelitis preparation for the purpose of protecting children in a family where a case of poliomyelitis has been reported. The administration of serum would probably be of more worth from the psychological point of view in allaying the fears of parents. Statistics would indicate that a second case in one family in interepidemic times is even rarer than the normal incidence for poliomyelitis in general. Flexner has already suggested the advisability of thus using convalescent serum.

More recently, Fairbrother (11), also aided by the Jeremiah Milbank gift has obtained an antipoliomyelitic horse serum which, subsequently, yielded a potent product.

SUMMARY

1. The horse, apparently itself unsusceptible to poliomyelitis, can be stimulated in certain cases but not all to the production of virucidal antibodies.

2. The virucidal potency of such immune serum can be raised to a point comparable to that of human convalescent serum and when concentrated and refined exhibits a four-fold increase in potency.

3. Such concentrates have proved effective in the prevention of paralysis in inoculated monkeys when given intraspinously before the onset of paralysis.

4. Treatment has been found more effective when therapeutic serums are given through the spinal route.

5. Pooled serum from "normal" adult donors has proved effective in neutralizing virus but its potency is approximately one-half that of convalescent serum.

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The results indicated in Chart 2 tend to show that the horse anti-body preparations are more effective when used intraspinaly than even greater amounts directed into the blood stream. Four monkeys out of six, treated intravenously survived as contrasted with the eight animals out of nine which failed to come down after intraspinal treatment even though instituted later in two cases.

One intraspinal injection is apparently as effective as two; material removed on the 2nd day after an injection is similar in physical properties at least to the injected globulin preparation.

		1 wk later	2 wks	3 wks	4 wks	5 wks	6 wks
421	20 cc. human convalescent	eee		Died, poliomyelitis			
422	20 cc. human convalescent			eee			Died, poliomyelitis
419	10 cc. horse globulin	eee			(Survived)	eee	(Survived)
420	10 cc. horse globulin		eee				(Survived)
411	5 cc. horse globulin				eee		(Survived)
403	1 cc. horse globulin				eee		Died, poliomyelitis
424	Control	eee		Died, poliomyelitis			

CHART 3

The fact that four out of five of the animals treated with human convalescent serum died may be attributed to the fact that the antibody concentration was not sufficient to prevent extension of the infection.

Observations on Prophylaxis

The value of the serum as a preventative can only be judged by the results of the few experiments shown in tabular form. 10 cc. of the globulin concentrate from an immunized horse was apparently responsible for protection over a period of at least 5 weeks. Twice the amount of human convalescent serum failed to protect for 3 weeks.

Girdwood (5), Lambert (6), French (7), Sutton (8).) Lister was unable to demonstrate agglutinins in the serum of animals or patients when the dose of vaccine was limited to hundreds of millions of cocci. In only one investigation of subcutaneous immunization was a study made of the appearance of protective substance in the patient's serum after administration of *Pneumococcus* Types I, II and III (Lambert). The results in this instance showed that protective substance appeared only against the type of pneumococcus which corresponded to that present in the patient's sputum.*

However, it was shown by G. and F. Klemperer (9) that the serum of patients after the crisis possessed curative powers in pneumococcus infection in the rabbit, and Neufeld (10) demonstrated agglutinins in patients convalescing from pneumonia.

That protective substance appeared in the serum of patients at the time of the crisis for the type of organism which was present in the sputum was demonstrated by Dochez (11), Clough (12) and others subsequently.

It is evident that the appearance of protective substance for the homologous type of pneumococcus may be ascribed to the natural course of the disease in recovered patients, and not necessarily to the injection of vaccine. It seemed to us necessary to inject heterologous types (*i.e.*, types of pneumococci not present in the sputum of the patient) and test for the appearance of protective substances against these types in order to demonstrate that active immunity may be induced by injection of vaccine during the course of lobar pneumonia. Thus, if specific protective substances against *Pneumococcus* Type II appear after injection of Type II vaccine in a patient suffering with Type I pneumonia, the initiation of this immunity might then be ascribed to the introduction of the vaccine.

The appearance of protective substances against the type of pneumococcus found in the patient's sputum following the injection of an homologous vaccine could only be ascribed to the vaccine if heterologous immunity was previously demonstrated under similar circumstances.

Another difficulty encountered is that the serum of normal individuals occasionally possesses demonstrable immunity against one or more types of pneumococcus (Neufeld and Haendel (13), Clough (14)). In certain of our patients the blood before injection of vaccine

* As these tables are unpublished, it is due to the kindness of Dr. Lambert that I have been allowed to inspect them and quote the results.

FACTORS INVOLVED IN THE PRODUCTION OF IMMUNITY WITH PNEUMOCOCCUS VACCINE

II. INDUCTION OF ACTIVE IMMUNITY DURING THE COURSE OF LOBAR PNEUMONIA

By ALVAN L. BARACH, M.D.

WITH THE TECHNICAL ASSISTANCE OF MAX SOROKA

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

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In a previous paper (1) the rate of development of immunity after injection of pneumococcus vaccine into normal animals was studied. In addition, the antigenic function of a vaccine made from the intact cell was compared with that derived from a watery extract of the cell free from formed elements. In each instance, a type-specific immunity was initiated. With the vaccine made from the intact cell, active immunity was demonstrated on the 3rd day after injection and, in the case of the filtrate, on the 4th day, in both instances increasing on the 5th and 6th days after administration of the antigen.

In the present study we administered pneumococcus vaccine to patients with lobar pneumonia to determine whether a similar state of active immunity could be induced during the course of the infection.

The literature concerned with the experimental production of pneumococcus immunity was reviewed in the first report. The attempts to induce active immunity in man during the course of lobar pneumonia may now be referred to.

Lister (2) injected pneumococcus vaccine intravenously into 40 South African natives suffering from lobar pneumonia, and concluded that the disease was favorably influenced. One patient who received an intravenous inoculation of 10 billion organisms the first dose, 20 billion the second dose and 30 billion the third dose, showed agglutinins in his serum 6 days subsequent to the first injection. Other investigators have employed subcutaneous injections of much smaller doses, such as 30 to 300 millions of diplococci, with reported lowering of the mortality rate of pneumonia as compared with control series. (Wright (3), Wynn (4).

The "Before," 3rd, 4th, 5th and 6th day bloods were tested by injecting the graded dilutions of culture plus 0.2 cc. of patient's serum into nine mice for each type: I, II and III. Thus, each experiment consists of the results in terms of survival (S) or death (number of hours after injection) on approximately 150 mice, including controls. Since the complete tabulation of all the experiments would occupy too large a space, the findings are condensed into tables which note the day on which protective substance appeared. This consisted of survival of one to three animals on one day followed by a larger number on succeeding days. A single survival that was not followed on other days by more survivals was reckoned as probably due to chance variation and not as evidence of passive immunity. Illustrative tables of individual experiments will be cited in each group.

RESULTS

Pneumococcus vaccine was administered in all to 37 patients, 29 of whom had pneumonia and 8 had miscellaneous diseases. The pneumococcus filtrate was injected intravenously in 7 patients, the vaccine of the whole organism was injected intravenously in 15 patients, intradermally in 13 patients and subcutaneously in 2 patients.

In the accompanying table (Table I), the results of the subcutaneous injection of 3 billion organisms of each of the three types, I, II and III, are shown. The patient was a man 36 years old, suffering from lobar pneumonia with Pneumococcus Type I in the sputum, a sterile blood culture and consolidation of the R. M. L. and R. L. L. He was treated on the 6th day of disease.

It is apparent from Table I that active immunity which was passively transmitted to mice began on the 3rd day and increased on the 4th, 5th and 6th days after injection in the case of Pneumococcus Type I, but was absent in Pneumococcus Types II and III. Since recovery in Type I pneumonia is followed by the spontaneous appearance of protective substance for that type, the presence of mouse survival 9 days after onset of illness for the homologous type of pneumococcus and the absence of mouse survival in the heterologous types indicate that the subcutaneous injection of pneumococcus vaccine was not effective in inducing active immunity as demonstrated by mouse protection during the 6 day period of the experiment. The second case was a woman of 33 years who had lobar pneumonia, Pneumococcus Type IV in the sputum, sterile blood culture and consolidation of the L.L.L. On the 3rd day of the disease she was given a subcutaneous injection of Pneumococcus II and III, 2.5 billion organisms of each type. No evidence of protective substance was apparent during the 6 day period after injection for Pneumococcus II or III.

The first case in the group treated by intravenous injection of vaccine (Case 1) is that of a man 49 years old who had chronic pulmonary tuberculosis, moderately advanced, and an acute serofibrinous pleurisy (which at first simulated pneumonia)

contained protective substances against one or two types of pneumococcus. These cases were therefore excluded.

Additional confirmation of the fact that heterologous protective substance does not spontaneously occur during the time interval covered by our experiments is obtained by the complete absence of heterologous immunity in two patients who were treated with subcutaneous immunization and a much larger group (14 cases) treated by intradermal injection.

Methods

The antigens were prepared as described in the previous paper (1). A virulent *Pneumococcus* Type I (or Type II or III) culture was passed through a mouse in preparation of the vaccine. 0.2 cc. of the heart's blood obtained from the animal immediately after death was inoculated into a test tube containing 5 cc. of beef-infusion broth with 2.5 per cent of human serum. After 8 hours' incubation, this was used to inoculate 250 cc. of similarly prepared broth. Incubation was carried on for 8 hours. The culture was then centrifuged and the supernatant broth poured off. Care was taken not to loosen the sedimented bacteria at the bottom of the centrifuge tube which was now carefully rinsed with normal saline to wash off all broth adhering to it. Normal salt solution was added to dilute the bacteria to a concentration of 10 billion organisms to 1 cc. of water. Sterilization was accomplished by heating the suspension for 1 hour at 60°C. This constituted the serum vaccine. For preparation of the filtrate the serum vaccine was shaken by hand for 10 minutes and centrifuged. The supernatant fluid was passed through a Berkefeld filter, bottled and placed in the ice-box. If sterile on culture, tricresol was added to a concentration of 0.3 per cent.

The filtrate was injected intravenously into patients in doses of 1 to 7 cc. Blood was withdrawn before injection and daily on the 3rd to the 6th day thereafter. An estimation of protective substance was made in the usual manner by injection of graded doses of virulent culture with 0.2 cc. of serum intraperitoneally into mice. The vaccine described above was injected intravenously in doses of 1 to 5 billion organisms. Intradermal injection was made in doses of 1 to 3 billion organisms (0.1 to 0.3 cc. of vaccine).

The test for protective substance was performed with maximally virulent organisms, the culture being generally fatal in 10^{-4} or 10^{-5} cc. in 24 to 40 hours. Controls (usually 3 to 5 mice for each type) were tested in every experiment. The dilution of culture employed was generally 0.001 cc., 0.0001 cc. and 0.00001 cc. Survival (noted S in tables) thus indicated protection against 100,000, 10,000 and 1,000 M. L. D. (minimal lethal doses), respectively. In instances in which the culture killed in a dilution of 10^{-4} , survival of an injection of 0.001 cc. indicated that the patient's serum protected a mouse against 1,000,000 M.L.D.

The next case cited (Case 4) is that of a man 28 years old who developed a post-operative bronchopneumonia involving the R.L.L. and L.L.L., in whose sputum were *Streptococcus haemolyticus*, staphylococcus and Friedländer bacillus, but no pneumococci. He was given 4 billion pneumococci, Type II, intravenously on the 6th day of disease, and 3 days later protective substance appeared in his blood for

TABLE II

Appearance of Protective Substance in Patient's Serum after Intravenous Injection of Pneumococcus Vaccine

Case 1.

Day after vaccination	Survival after injection of Pneumococcus I culture plus 0.2 cc. patient's serum			Survival after injection of Pneumococcus II culture plus 0.2 cc. patient's serum			Survival after injection of Pneumococcus III culture plus 0.2 cc. patient's serum		
	0.001	0.0001	0.00001	0.001	0.0001	0.00001	0.001	0.0001	0.00001
Before	20	30	30	40	40	40	40	40	40
	30	30	30	40	40	40	40	40	40
	30	30	30	40	40	40	40	40	40
3rd	20	35	30	30	40	40	40	40	40
	30	32	30	30	40	40	40	40	40
	30	35	30	30			40	40	40
4th	35	40	40	30	40	40	40	60	40
	40	40	40	30	40	S	40	80	S
	40	40	40	30	S	S	60	S	S
5th	20	40	40	60	S	S	S	40	40
	20	40	40	60	S	S	S	S	60
	40	40	48	S	S	S	S	S	S
6th	20	48	48	120	S	S	60	40	S
	48	48	48	S	S	S	60	60	S
	48	48	48	S	S	S	40	60	S

Controls
 0.00001 }
 0.000001 } 40 hrs.
 0.0000001 }

Controls
 0.00001 }
 0.000001 } 40 hrs.
 0.0000001 }

Controls
 0.00001 } 40 hrs.

Diagnosis.—Acute serofibrinous pleurisy. Patient was given pneumococcus vaccine intravenously, 2 billion organisms of each type, I, II and III.

Type II (continuing on the 4th, 5th and 6th days) but none developed for Types I and III.

In this case of pneumonia without pneumococcus infection, immunity was developed specifically for the type of pneumococcus injected and not for the other two types during the febrile period of the disease. It began 3 days after intravenous injection of the vaccine.

In the case shown in Table IV (Case 6), the patient was a man 47 years old who

of 2 days' duration. He was given 2 billion organisms of each type, I, II and III, intravenously.

As seen in Table II, protective substance was present on 4th, 5th and 6th days after injection for *Pneumococcus* Types II and III, and absent for *Pneumococcus*

TABLE I

Appearance of Protective Substance in Patient's Serum after Subcutaneous Injection of Pneumococcus Vaccine

Case 36.

Day after vaccination	Survival after injection of <i>Pneumococcus</i> I culture plus 0.2 cc. patient's serum			Survival after injection of <i>Pneumococcus</i> II culture plus 0.2 cc. patient's serum			Survival after injection of <i>Pneumococcus</i> III culture plus 0.2 cc. patient's serum		
	0.001	0.0001	0.00001	0.001	0.0001	0.00001	0.001	0.0001	0.00001
Before	24	24	40	24	24	24	24	24	24
	24	24	72	24	24	24	24	24	24
	24	24	72	24	24	24	24	24	24
3rd	24	24	40	24	24	24	24	24	24
	24	24	72	24	24	40	24	24	24
	40	72	S	24	24	24	24	24	24
4th	24	S	S	24	24	24	24	24	24
	24	S	S	24	24	24	24	24	24
	24	S	S	24	24	24	24	24	24
5th	24	24	40	24	24	24	24	24	24
	24	24	S	24	24	24	24	24	24
	24	S	S	24	24	24	24	S	24
6th	24	S	24	24	24	24	24	24	24
	40	S	40	24	24	24	24	24	24
	S	S	S	24	72	40	24	24	24

Controls

0.00001 }
0.000001 } 40 hrs.
0.0000001 }

Controls

0.00001 }
0.000001 }
0.0000001 } 24 hrs.
0.00000001 }
0.000000001 }

Controls

0.00000001 }
0.000000001 } 40 hrs.

Diagnosis.—Lobar pneumonia, *Pneumococcus* Type I. Patient was given pneumococcus vaccine subcutaneously, 3 billion organisms of each type, I, II and III.

Type I. Since the patient was not suffering from pneumonia or pneumococcus infection, the appearance of mouse survival leads to the conclusion that an immunity against *Pneumococcus* Types II and III was produced 4 days after intravenous injection of the corresponding vaccine, although no response was elicited in the case of Type I.

10^{-7} or 10,000 M.L.D.). In this instance, definite heterologous immunity was developed during the course of pneumococcus pneumonia.

The case presented in Table V (Case 12), is a man 40 years old who had lobar pneumonia, with *Pneumococcus* Type I in the sputum, negative blood culture

TABLE IV

Appearance of Protective Substance in Patient's Serum after Intravenous Injection of Pneumococcus Vaccine

Case 6.

Day after vaccination	Survival after injection of <i>Pneumococcus</i> II culture plus 0.2 cc. patient's serum			Survival after injection of <i>Pneumococcus</i> III culture plus 0.2 cc. patient's serum		
	0.01	0.001	0.0001	0.01	0.001	0.0001
Before	24	24	24	24	24	40
	24	25	25	24	40	40
	24	25	40	40	50	40
3rd	24	24	24	24	24	25
	24	24	40	40	25	40
	24	25	40	40	25	40
4th	24	24	S	24	25	40
	24	24	S	24	40	40
	24	24	S	25	40	40
5th	24	24	24	24	24	24
	24	S	S	24	24	40
	24	S	S	40	24	40
6th	40	S	S	24	40	40
	S	S	S	24	S	S
	S	S	S	24	S	S

Controls

0.0001 24 hrs.
 0.00001 25 hrs.
 0.000001 28 hrs.
 0.0000001 30 hrs.
 0.00000001 35 hrs.
 0.000000001 40 hrs.

Controls

0.0001 24 hrs.
 0.00001 25 hrs.
 0.000001 28 hrs.
 0.0000001 30 hrs.

Diagnosis.—Lobar pneumonia, *Pneumococcus* Type IV. Patient received 4 billion organisms of each Type II and III vaccine.

and consolidation of the R.M.L. He received 3 billion pneumococci intravenously of each Type I and II on the 3rd day of illness.

As seen in Table V, protective substance developed for the homologous Type I on the 3rd day after injection, and gradually increased on the 4th, 5th and 6th days; for the heterologous Type II on the 4th day, increasing on the 5th and 6th

had lobar pneumonia, *Pneumococcus* Type IV in his sputum, negative blood culture and consolidation of the R.L.L. He was given 4 billion pneumococci of each Type II and III in two doses on the 5th day of disease.

It will be observed that protective substance appeared on the 4th day after injection for Type II pneumococcus, and increased markedly on the 5th and 6th

TABLE III

Appearance of Protective Substance in Patient's Serum after Intravenous Injection of Pneumococcus Vaccine

Case 4.

Day after vaccination	Survival after injection of <i>Pneumococcus</i> I culture plus 0.2 cc. patient's serum			Survival after injection of <i>Pneumococcus</i> II culture plus 0.2 cc. patient's serum			Survival after injection of <i>Pneumococcus</i> III culture plus 0.2 cc. patient's serum		
	0.001	0.0001	0.00001	0.001	0.0001	0.00001	0.001	0.0001	0.00001
Before	24	24	24	24	40	40	24	40	40
	40	40	40	40	40	40	24	40	40
	40	40	40	40	100	40	40	40	40
3rd	24	24	40	24	100	S	24	40	40
	40	40	40	40	S	S	24	40	40
	40	40	40	40	S	S	24	40	40
4th	24	20	24	24	40	40	24	24	24
	24	40	40	40	40	S	40	24	24
	24	40	40	40	40	S	40	40	40
5th	24	40	40	24	40	S	24	40	40
	40	40	40	40	S	S	40	40	40
	40	40	40	40	S	S	40	40	40
6th	24	40	40	24	40	40	24	40	40
	24	40	40	24	40	S	24	40	40
	24	40	40	40	S	S	40	40	40

Controls

0.00001 }
 0.000001 } 40 hrs.
 0.0000001 }
 0.00000001 }

Controls

0.00001 }
 0.000001 } 40 hrs.
 0.0000001 }

Controls

0.00001 }
 0.000001 } 40 hrs.
 0.0000001 }

Diagnosis.—Post-operative bronchopneumonia. Sputum contained *Streptococcus haemolyticus*, staphylococcus and Friedländer bacillus. Patient received 4 billion *Pneumococcus* Type II intravenously.

day. (On the latter day, 0.2 cc. of his serum protected against 0.01 cc. of culture with a virulence of 10^{-7} , approximately 10,000,000 M.L.D.) In the case of Type III, protective substance appeared on the 6th day after injection and was not as great, (0.2 cc. serum protecting against 0.001 cc. of a culture with a virulence of

which homologous vaccine (composed of pneumococci of the same type as that in the sputum) was given (Table IX).

TABLE VI

Appearance of Protective Substance after Intravenous Injection of Pneumococcus Vaccine: Heterologous Type Experiments

Case No.	Organism in patient's sputum	Type of pneumococcus vaccine administered	Day after injection on which protective substance appeared
10	Pneumococcus II	I	6
13	—	I	4
15	Hemolytic streptococci	I	6
1	Tubercle bacillus	I	Negative
2	—	I	Negative
5	Pneumococcus III	I	Negative
1	Tubercle bacillus	II	4
2	Pneumococcus IV	II	3
3	Pneumococcus IV	II	5
4	Friedländer bacillus	II	3
5	Pneumococcus III	II	6
6	Pneumococcus IV	II	4
7	Pneumococcus IV	II	4
8	Pneumococcus I	II	6
9	Pneumococcus I	II	3
11	Pneumococcus I	II	3
12	Pneumococcus I	II	4
13	—	II	3
14	Pneumococcus I	II	4
15	Hemolytic streptococci	II	5
1	Tubercle bacillus	III	4
6	Pneumococcus IV	III	6
7	Pneumococcus IV	III	6

Total experiments, 23.

Total cases showing protective substance 20, or 87 per cent.

Average day of onset of 3 cases injected with Type I vaccine, 5.3 days.

Average day of onset of 14 cases injected with Type II vaccine, 4.1 days.

Average day of onset of 3 cases injected with Type III vaccine, 5.3 days.

Combined average day of onset of 20 cases injected with Type I, II or III vaccine, 4.4 days.

In the accompanying table (Table VI), there were 23 instances in which a heterologous vaccine was given intravenously. Of these 20, or 87 per cent, developed protective substance for the organism in-

day after injection. The blood before injection contained protective substance for Type III pneumococcus, with increase in degree on the 3rd to the 6th days after injection.

Tables I to V illustrate the various types of response to intravenous injection of pneumococcus vaccine. The results in this series

TABLE V

Appearance of Protective Substance in Patient's Serum after Intravenous Injection of Pneumococcus Vaccine

Case 12.

Day after vaccination	Survival after injection of Pneumococcus I culture plus 0.2 cc. patient's serum			Survival after injection of Pneumococcus II culture plus 0.2 cc. patient's serum			Survival after injection of Pneumococcus III culture plus 0.2 cc. patient's serum		
	0.001	0.0001	0.00001	0.001	0.0001	0.00001	0.001	0.0001	0.00001
Before	24	24	48	24	24	24	24	48	S
	24	24	48	24	24	24	48	48	S
	48	48	48	24	24	24	48	48	S
3rd	24	24	24	24	24	24	24	24	48
	24	72	S	24	24	24	24	S	48
	S	48	S	24	24	24	24	S	S
4th	24	24	S	24	24	48	24	72	48
	24	24	S	48	24	48	24	48	S
	48	48	S	48	24	S	24	S	S
5th	24	48	72	24	24	24	24	24	S
	24	S	48	24	48	S	48	48	S
	24	S	S	24	48	S	48	48	S
6th	24	S	S	24	24	72	24	S	S
	24	S	S	S	24	S	48	S	S
	24	S	S	S	72	S	S	S	S

Controls

0.00001 24 hrs.
0.000001 48 hrs.
0.0000001 48 hrs.
0.00000001 48 hrs.

Controls

0.00001 48 hrs.
0.000001 48 hrs.
0.0000001 48 hrs.
0.00000001 24 hrs.
0.000000001 48 hrs.

Controls

0.00001 48 hrs.
0.000001 72 hrs.

Diagnosis.—Lobar pneumonia. Sputum contained *Pneumococcus* Type I. Patient was given an intravenous injection of pneumococcus vaccine, 3 billion organisms of each Type I and II.

have been divided into a group in which heterologous vaccine (i.e., composed of organisms of a different type than that present in the sputum) was administered intravenously (Table VII), and a group in

injection are presented. In 8, or 89 per cent, protective substance appeared during the period under observation. The average day of onset of active immunity was 5.6 days after injection; 5.5 days for Type II filtrate and 5.7 for Type I filtrate.

It is of interest to note that the intravenous injection of vaccine resulted in the appearance of protective substance 1.2 days earlier than the filtrate, being approximately the same difference that was found in the animal experiments reported in the previous paper (1).

TABLE VIII

Appearance of Protective Substance after Intravenous Injection of Pneumococcus Vaccine and Filtrate: Homologous Type Experiments

Case No.	Organism in patient's sputum	Type of pneumococcus administered. Vaccine or filtrate	Day after injection on which protective substance appeared
10	Pneumococcus II	Vaccine II	5
12	Pneumococcus I	Vaccine I	3
14	Pneumococcus I	Vaccine I	3
18	Pneumococcus II	Filtrate II	3
19	Pneumococcus II	Filtrate II	6
22	Pneumococcus II	Filtrate II	4
22	Pneumococcus I	Filtrate I	4

Total cases showing protective substance, 7, or 100 per cent.

Average day of onset of 3 cases injected with vaccine, 3.7 days.

Average day of onset of 4 cases injected with filtrate, 4.2 days.

Combined average day of onset of 7 cases injected with vaccine or filtrate, 4.0 days.

In Table VIII, the findings after intravenous injection of homologous vaccine and filtrate are summarized. The average day of onset of passively transmitted immunity in the case of 3 vaccine cases was 3.7 days, in the 4 filtrate cases, 4.2 days. In both instances immunity appeared earlier than in the corresponding heterologous cases. That this might be due to the spontaneous development of protective substances and not to the vaccine has been previously emphasized.

Two illustrative cases of intravenous filtrate injection will be mentioned.

jected. The average day of onset of active immunity as demonstrated by passively transmitted protection to mice was 4.4 days after injection; 4.1 days for 14 cases of *Pneumococcus* Type II, and 5.3 days for 3 cases of Type I and 3 cases of Type III. Patients without pneumococcus infection were included in this group in order to add further evidence that heterologous immunity was due to the

TABLE VII

Appearance of Protective Substance after Intravenous Injection of Pneumococcus Filtrate: Heterologous Type Experiments

Case No.	Organism in patient's sputum	Type of pneumococcus vaccine administered	Day after injection on which protective substance appeared
16	<i>Pneumococcus</i> IV	II	6
17	<i>Pneumococcus</i> III	II	6
20	<i>Pneumococcus</i> IV	II	6
21	<i>Pneumococcus</i> IV	II	4
16	<i>Pneumococcus</i> IV	I	6
17	<i>Pneumococcus</i> III	I	6
18	<i>Pneumococcus</i> II	I	5
19	<i>Pneumococcus</i> II	I	6
20	<i>Pneumococcus</i> IV	I	Negative

Total experiments, 9.

Total cases showing protective substance, 8, or 89 per cent.

Average day of onset of protective substance of 4 cases injected with Type II vaccine, 5.5 days.

Average day of onset of protective substance of 4 cases injected with Type I vaccine, 5.7 days.

Combined average day of onset of protective substance of 8 cases injected with Types I or II vaccine, 5.6 days.

introduction of vaccine and not to the spontaneous appearance of pneumococcus protective substance.

The results of the intravenous injection of pneumococcus filtrate have been summarized in two tables, (1) the appearance of protective substance after administration of heterologous filtrate (Table VII), and (2) appearance of protective substance after administration of homologous filtrate (Table VIII).

In the former table, (Table VII), 9 instances of heterologous filtrate

In the first case (Case 17), a man of 38 years had lobar pneumonia with *Pneumococcus* Type III in the sputum, sterile blood culture and involvement of the L.L.L. On the 2nd day of illness, 16 cc. of *Pneumococcus* Type I and II filtrate were injected intravenously in 5 doses in 24 hours.

TABLE X

Appearance of Protective Substance after Intravenous Injection of Antigenic Filtrate
Case 18.

Day after injection of filtrate	Survival after injection of test culture					
	<i>Pneumococcus</i> Type I			<i>Pneumococcus</i> Type II		
	0.001	0.0001	0.00001	0.001	0.0001	0.00001
Before	20	40	40	20	20	20
	20	40	40	20	20	20
	40	40S	40	20	20	30
3rd	20	20	20	20	20	20
	20	40	20	20	20	20
	20	40	40	20	S	20
4th	20	40	20	20	S	S
	20	40	40	40	S	S
	40	40	40	40	S	S
5th	20	40	40	20	S	S
	20	40	S	20	S	S
	40	90	S	40	S	S
6th	S	S	40	20	S	S
	S	S	S	20	S	S
	S	S	S	S	S	S
8th	40	S	40	S	S	S
	90	S	S	S	S	S
	S	S	S	S	S	S

Controls
 0.000001
 0.0000001
 0.00000001
 0.000000001 } 40 hrs.

Controls
 0.000001
 0.0000001
 0.00000001 } 40 hrs.

Diagnosis.—Lobar pneumonia. Sputum contained *Pneumococcus* Type II. Patient received intravenously 17 cc. of *Pneumococcus* I and II filtrate in 5 doses.

As seen in Table IX, protective substances appeared on the 6th day (and were still present on the 14th day) after injection against *Pneumococcus* Types I and II. In this instance of immunity induced against two heterologous types of pneumococcus, the filtrate appeared clearly to be the cause of the appearance of protective substance.

TABLE IX

Appearance of Protective Substance after Intravenous Injection of Antigen Filtrate
Case 17.

Day after injection of filtrate	Survival after injection of test culture					
	Pneumococcus Type I			Pneumococcus Type II		
	0.001	0.0001	0 00001	0.001	0.0001	0.00001
Before	20	20	25	20	20	20
	20	25	40	20	20	25
	40	40	90	20	20	25
1st	20	20	25	20	20	24
	20	20	40	20	20	20
	40	40	70	20	20	20
2nd	20	25	20	20	20	20
	20	40	40	20	20	20
	75	40	40	20	20	20
3rd	20	20	24	20	20	20
	20	20	40	20	20	20
	40	40	40	20	20	S
4th	20	20	40	20	20	20
	20	20	40	20	20	20
	20	25	40	20	20	20
5th	20	25	40	20	20	20
	20	24	40	20	20	24
	25	40	96	20	40	24
6th	30	24	S	20	40	40
	20	20	S	20	40	S
	20	96	S	24	S	S
2 weeks	SS		S			
	30	S	S		S	S
	30	S	S			S
	60	S	S		S	

Controls

0.001 20 hrs.
0.0001 20 hrs.
0.00001 25 hrs.
0.000001 40 hrs.
0.0000001 40 hrs.
0 00000001 40 hrs.

Controls

0.001 20 hrs.
0.0001 20 hrs.
0 00001 35 hrs.
0.000001 40 hrs.
0.0000001 40 hrs.
0.00000001 40 hrs.

Diagnosis.—Lobar pneumonia. Pneumococcus Type III in sputum. Patient received 17 cc. of Pneumococcus I and II filtrate.

The results of the intradermal injection of vaccine may now be summarized.

Of 24 instances in which heterologous pneumococcus vaccine was injected intradermally, 10, or 42 per cent, showed a positive response,

TABLE XII

Appearance of Protective Substance in Patient's Serum after Intradermal Injection of Pneumococcus Vaccine

Case 27.

Day after vaccination	Survival after injection of Pneumococcus I culture plus 0.2 cc. patient's serum			Survival after injection of Pneumococcus II culture plus 0.2 cc. patient's serum			Survival after injection of Pneumococcus III culture plus 0.2 cc. patient's serum		
	0.001	0.0001	0.00001	0.001	0.0001	0.00001	0.001	0.0001	0.00001
Before	40	40	40	20	40	20	20	40	40
	40	40	40	40	40	40	40	40	40
		40	40		40	40		40	
3rd	20	40	40	20	20	20	40	20	40
	40	40	40	20	40	40	40	40	40
		40	40		40	40		40	40
4th	20	20	40	20	20	90	40	40	40
	20	40	40	20	20	40	40	40	40
		40	40		40	40		40	80
5th	40	40	40	20	40	20	40	40	40
	40	40	40	40	40	20	40	40	40
		40	40		40	40		S	40
6th	48	48	48	20	20	40	40	40	40
	45	48	48	20	40	60	40	S	40
		60	48		40	60	40	S	80

Controls
 0.00001 20 hrs.
 0.000001 25 hrs.
 0.0000001 30 hrs.
 0.00000001 40 hrs.
 0.000000001 45 hrs.

Controls
 0.000001 40 hrs.
 0.0000001 40 hrs.
 0.00000001 40 hrs.
 0.000000001 80 hrs.

Controls
 0.00001 40 hrs.
 0.000001 40 hrs.
 0.0000001 40 hrs.
 0.00000001 40 hrs.

Diagnosis.—Lobar pneumonia. Sputum contained Pneumococcus Type IV. Patient received an intradermal injection of pneumococcus vaccine, 2 billions of organisms of each Type I, II and III.

and had an average day of onset of immunity of 4.5 days after injection. The degree of immunity as well as its regularity is much less marked than in intravenous injection. One illustrative case of this group will be cited:

The second case (Case 18), was a woman 28 years old who had lobar pneumonia, with *Pneumococcus* Type II in sputum, sterile blood culture and consolidation of the R.L.L. On the 5th day of disease, 17 cc. of *Pneumococcus* Type I and II filtrate were injected intravenously in 5 doses.

TABLE XI

Appearance of Protective Substance after Intradermal Injection of Pneumococcus Vaccine

Heterologous Cases

Case No.	Organism in patient's sputum	Type of pneumococcus administered	Day after injection on which protective substance appeared
23	<i>Pneumococcus</i> I	II	5
24	<i>Pneumococcus</i> I	II	6
29	<i>Pneumococcus</i> I	II	4
23	<i>Pneumococcus</i> I	III	3
29	<i>Pneumococcus</i> I	III	4
26	<i>Pneumococcus</i> IV	II	4
27	<i>Pneumococcus</i> IV	III	5
25	<i>Pneumococcus</i> IV	II	5
28	Tubercle bacillus	II	5
34	—	III	4
33	—	III	Negative
32	—	II	Negative
32	—	II	Negative
33	—	II	Negative
33	—	I	Negative
34	—	II	Negative
34	—	I	Negative
35	<i>Pneumococcus</i> III	II	Negative
24	<i>Pneumococcus</i> I	III	Negative
27	<i>Pneumococcus</i> IV	II	Negative
25	<i>Pneumococcus</i> IV	III	Negative
26	<i>Pneumococcus</i> IV	III	Negative
27	<i>Pneumococcus</i> IV	I	Negative
35	<i>Pneumococcus</i> III	I	Negative

10 of 24 cases, or 42 per cent, developed protective substance after intradermal vaccination. Average day of onset, 4.5 days after injection.

As noted in Table X, protective substances appeared on the 3rd day after injection in the case of the homologous Type II filtrate and 5 days after injection in the heterologous Type I filtrate.

lyzed pneumococci in the lung which escape in relatively small numbers into the blood stream might therefore provide a less efficient stimulus for the production of protective antibodies than the introduction into the vascular system of large numbers of whole dead pneumococcus organisms.

The intravenous injection of pneumococcus vaccine in human beings was followed by the appearance of specific protective substances in their serum 4 to 5 days after administration. This was demonstrated in cases of miscellaneous disease without lung involvement, in pulmonary inflammation without pneumococcus infection and in lobar

TABLE XIII

Temperature Range on Day Protective Substance Appeared after Intravenous Injection of Pneumococcus Vaccine

Case No.	Temperature range
1	100.8–103.8°
4	100.0–103.0°
10	100.4–102.0°
12	101.0–103.0°
13	100.2–104.0°
3	98.2– 99.8°
6	98.2– 99.2°
7	98.8– 99.4°
8	98.6– 99.6°
9	99.6–104.0°
11	98.0– 99.2°
14	99.4–100.5°

pneumonia caused by the pneumococcus. By the injection of heterologous types of pneumococcus, it was possible to evoke specific protective substance for the corresponding types during the febrile course of pneumonia. In this discussion, emphasis has been placed on the development of specific protective substance during the course of acute pneumonia. Although all patients were injected in the presence of fever, in many instances the temperature had declined to normal or nearly so when antibodies appeared in the serum. It was possible, however, to demonstrate the production of specific protective substance during the febrile period. In two cases of intravenous filtrate injection and five cases of intravenous vaccine injection, antibodies

The patient (Case 27) was a man 38 years old who had lobar pneumonia, with *Pneumococcus* Type IV in the sputum, negative blood cultures and consolidation of the R.L.L. He was given an intradermal injection of 2 billion pneumococci of each Type I, II and III on the 3rd day of disease.

As will be seen in Table XII, no immunity appeared for *Pneumococcus* Types I and II, but one survival was present for Type III 5 days after injection and two survivals 6 days after injection. A slight though definite instance of heterologous pneumococcus immunity seemed to result from intradermal vaccination.

DISCUSSION

Inasmuch as pneumonia is a self-limited disease ending with recovery or death, generally between the 7th and 12th days after onset, 3 to 5 days are frequently afforded in which an attempt can be made to produce active immunity before the natural termination of the disease takes place. The previous study of the rate of development of pneumococcus immunity in animals was suggested by Dochez (11) in order to determine whether active immunity to the pneumococcus could be developed in a sufficiently short space of time as to make the injection of vaccine a therapeutic measure in lobar pneumonia. In addition, the life of the pneumonia patient, in the author's experience (15), was at times prolonged by oxygen treatment, a circumstance that seemed to increase the possibilities of vaccine treatment in an individual case.

That a rational basis for applying vaccine therapy in lobar pneumonia is now present has been recently emphasized by Zinsser (16). He states that autoimmunization from the patient's own lesion may be inefficient for two reasons: (1) the fact that considerable autolysis of the pneumococcus takes place in the lung (17), and (2) that autolyzed pneumococci possess little antigenic value, and are especially deficient in the capacity to evoke type-specific antibodies (18). Since active immunity in animals within a 3 to 5 day period was produced in the study above referred to, and similar results were reported by Goodner (19) at the same time, a rational basis for attempting vaccine therapy in lobar pneumonia seemed justified.

It may also be noted in this connection that the site of the antibody-producing tissues is thought to be in the capillary and lymphatic endothelium and other reticulo-endothelial cells (Boone (20) and Manwaring (21)). The presence of large numbers of partially auto-

ferable or passive immunity. A state of active immunity against the pneumococcus may be produced without giving evidence of its existence by the presence of protective antibodies in the serum. This has been demonstrated by several workers, particularly by Cecil and Steffen (24), who were able to immunize monkeys against pneumonia by three subcutaneous injections of pneumococcus vaccine at weekly intervals. Numerous instances occurred in which the monkeys survived lethal doses of virulent cultures without possessing either serum agglutinins or protective substance. Active immunity, however, was not produced within the first 6 days after subcutaneous injection of vaccine. Immunization of rabbits with Type III pneumococcus may also be effective in producing active immunity against infection in the absence of type-specific protective antibodies (Tillett (25)).

The results of intradermal injection of vaccine are confusing. Although the majority of cases were negative, apparent instances of induction of active immunity did occur. The degree and regularity of response in an individual case was much less marked than in the case of intravenous injection of vaccine. The findings were sufficiently indefinite as to permit no conclusion as to the effect of the intradermal injection of vaccine in the production of pneumococcus immunity.

The clinical results have not been emphasized in this series, since the production of *heterologous* protective substance seemed necessary to demonstrate that an immunity may actually be initiated during the febrile period of pneumonia by the intravenous injection of a suitable pneumococcus antigen. There were 20 cases of pneumonia, 17 lobar pneumonia and 3 bronchopneumonia, who received an intravenous injection of pneumococcus vaccine or filtrate. Of these, 2 died. One was a patient who had a *Pneumococcus* Type IV in the sputum with a persistent Type IV pneumococcus bacteremia. She received an intravenous injection of *Pneumococcus* Type I and II filtrate. The other death occurred in a patient who had a lobar pneumonia due to *Pneumococcus* Type II with a blood stream infection with *Pneumococcus* Type II. The patient was injected with *Pneumococcus* Type I and II vaccine intravenously. Of 9 patients with pneumonia, 7 lobar and 2 bronchopneumonia, who received an intradermal injection of vaccine, 1 died. Two patients who received a subcutaneous injection of vaccine recovered. Eight patients with

were present in the serum while the acute febrile stage of the pneumonia was in progress. A table (Table XIII) of the temperature range in the cases who were given intravenous vaccine shows the degree of fever on the day when protective substances first appeared. The first five developed antibodies while definite fever was still present. The remainder were in a nearly afebrile condition when protective antibodies were demonstrated. No case was considered as belonging to the febrile group if the temperature at any time went below 100°. It seems evident, therefore, that active immunity may be induced during the febrile course of pneumonia as a result of intravenous injection of pneumococcus vaccine.

The appearance of protective substance after injection of homologous vaccine appeared almost a day earlier than that which followed heterologous vaccine, but it is not possible to affirm that the injection of the vaccine in an individual instance was responsible for the appearance of homologous protective substance. Our experiments indicate, however, that immunity may generally be established 4 to 5 days after intravenous injection of pneumococcus vaccine. In cases of pneumonia that are seen early in the disease, the capacity to evoke type-specific protective substances in 4 or 5 days may make this procedure of therapeutic value.

Subcutaneous injection of vaccine in two cases of pneumonia and in ten normal donors* did not produce protective substance within this period. In Whitmore's (22) experiments with pneumococcus vaccine injected subcutaneously in normal human beings, protective substances did not appear in the serum until 8 days after injection. The same result was reported by Cecil and Austin (23). The results of Lambert in his own series (referred to in the early part of the paper) showed no heterologous protection. Although clinically a lowered mortality was present in the treated group of Lambert, the factor responsible for the favorable result does not appear in immunological studies so far reported.

It must be remembered, however, that the object of injection of vaccine is to produce active immunity, and that the measurement of protective substance in the serum of the patients records only trans-

* The findings on donors is part of a separate report on immune transfusion.

patients and in only one instance did a foreign protein reaction occur. In this case, a chill of moderate severity was followed by a defervescence of fever in 8 hours with a return of the temperature to its previous level 12 hours later.

The number of cases of pneumonia treated by the intravenous injection of homologous pneumococcus vaccine is too small to permit conclusions as to its therapeutic value at this time.

SUMMARY

1. Pneumococcus vaccine was administered to 29 patients with pneumonia to determine whether a state of immunity could be induced during the course of the disease. Twenty patients received an intravenous injection of pneumococcus vaccine or pneumococcus filtrate. Nine pneumonia patients received an intradermal injection of vaccine. Eight patients with miscellaneous disease received an intravenous or intradermal injection of pneumococcus vaccine.

2. Of 23 tests in which the serum of the patient was studied for the appearance of protective substance after intravenous injection of heterologous pneumococcus vaccine, 20 or 87 per cent showed a positive response within 6 days after the administration of the antigen. The average day of onset was 4.4 days after injection.

3. Of 9 tests of the same character following the intravenous injection of pneumococcus filtrate, 8 or 89 per cent showed a positive response. The average day of onset of protective substance was 5.6 days after injection.

4. The appearance of specific protective substance following heterologous injection of pneumococcus vaccine appeared to be due to the introduction of the vaccine and not to the natural course of the disease, as was shown by negative control experiments.

5. Of 24 tests with intradermal injection of vaccine, 10 or 42 per cent developed slight protective substance of irregular degree 4.5 days after injection.

6. No immediate reactions were observed following the intravenous or intradermal injection of pneumococcus vaccine. One chill occurred after injection of pneumococcus filtrate. Of 20 cases with intravenous injection of pneumococcus vaccine or filtrate, 2 died of their disease, one a case in which homologous vaccine was used and one in which heterologous vaccine was administered.

miscellaneous disease were treated, of whom one subsequently died of causes unrelated to vaccine injection. Four cases were entirely excluded because they died before observations on the appearance of protective substance could be made. Their clinical course gave no indication of having been in any way affected by the introduction of vaccine.

TABLE XIV

Clinical Data on Pneumonia Patients Who Received an Intravenous Injection of Pneumococcus Antigen

Case No.	Diagnosis	Organism in sputum	Day of disease injected	Pneumococcus antigen employed	Outcome
5	Lobar pneumonia	Pneumococcus III	3	Vaccine I, II	S
6	Lobar pneumonia	Pneumococcus IV	5	Vaccine II, III	S
7	Lobar pneumonia	Pneumococcus IV	4	Vaccine II, III	S
8	Lobar pneumonia	Pneumococcus I	3	Vaccine I	S
9	Lobar pneumonia	Pneumococcus I	2	Vaccine II	S
10	Lobar pneumonia	Pneumococcus II	5	Vaccine I, II	D
11	Lobar pneumonia	Pneumococcus I	2	Vaccine II, III	S
12	Lobar pneumonia	Pneumococcus I	3	Vaccine I, II	S
13	Lobar pneumonia	—	7	Vaccine I, II	S
14	Lobar pneumonia	Pneumococcus I	6	Vaccine I, II	S
16	Lobar pneumonia	Pneumococcus IV	1	Filtrate I, II	S
17	Lobar pneumonia	Pneumococcus III	2	Filtrate I, II	S
18	Lobar pneumonia	Pneumococcus II	5	Filtrate I, II	S
19	Lobar pneumonia	Pneumococcus II	3	Filtrate I, II	S
20	Lobar pneumonia	Pneumococcus II	4	Filtrate I, II	S
21	Lobar pneumonia	Pneumococcus IV	1	Filtrate I, II	D
22	Lobar pneumonia	Pneumococcus I, II	4	Filtrate I, II	S
2	Bronchopneumonia	—	—	Vaccine I, II	S
3	Bronchopneumonia	Pneumococcus IV	3	Vaccine I, II	S
4	Bronchopneumonia	<i>Streptococcus haemolyticus</i> , Friedländer bacillus	6	Vaccine II	S

None of the patients who received an intravenous or intradermal injection of pneumococcus vaccine experienced any reaction. There was no change in pulse, respiration or temperature. No consistent alteration of the clinical course of the disease was discerned. The pneumococcus filtrate was administered intravenously 28 times to 7

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7. Conclusions concerning the therapeutic value of the introduction of pneumococcus vaccine in pneumonia must await further investigation. These studies demonstrate that specific protective substances generally appear 4 to 5 days after intravenous injection of pneumococcus vaccine during the course of lobar or bronchopneumonia.

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resorption and marrow fibrosis, depending on the daily dose and the length of treatment; bone resorption occurred in young animals even in the absence of hypercalcemia; hypercalcemia was found after the largest doses, but toxic symptoms (with hyperphosphatemia) were absent even after the largest doses when these had been preceded by treatment with smaller doses, some compensation having apparently been established by this means.

A procedure similar in principle was employed to produce chronic hyperparathyroidism in the dog. The dog, as is well known, is very sensitive to moderate doses of parathormone as well as to overdosage (5). While this sensitiveness has been an advantage in studies of acute hyperparathyroidism, it has limited the dog's usefulness in prolonged experiments. Greenwald and Gross (6) found negative calcium and phosphorus balances (in adult dogs after a prolonged period of parathormone treatment) of such magnitude as to be clearly due to bone depletion, though this was not demonstrable by the X-ray (7). Histological studies were not made. The dosage had necessarily been limited by the need of avoiding fatal effects.³

It was our aim not only to induce a condition of chronic hyperparathyroidism, but also to maintain this condition for periods as long as possible, in order to allow the most definite development of the bone lesions. We preferred young animals on an *a priori* assumption that bone changes may be expected to be more pronounced in an actively growing animal. This assumption has found its experimental justification in the standard methods for the production of rickets. The more pronounced effects of parathormone upon the serum calcium which we have observed in actively growing animals may be related to the greater availability of their calcium reserves.

After having established the limits of safe and effective parathormone dosage at different levels of calcium intake (9), we studied the responses of serum calcium and phosphorus to single parathormone

³ The results of Morgan and Garrison (8), showing the influence of vitamin D on response to parathormone, were reported after most of the present study had been completed. Among their dogs there was one 3.5 months old at the beginning of the experiment, on an adequate diet, which received repeated parathormone treatment for 5 weeks. They did not report on the mineral metabolism of this dog, or on the bone changes.

PSYCHOPATHIC HOSPITAL

PARATHORMONE DOSAGE AND SERUM CALCIUM AND PHOSPHORUS IN EXPERIMENTAL CHRONIC HYPERPARATHYROIDISM LEADING TO OSTITIS FIBROSA¹

BY AARON BODANSKY, PH.D., AND HENRY L. JAFFE, M.D.

(From the Laboratory Division of the Hospital for Joint Diseases, New York)

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Clinical *ostitis fibrosa cystica* (von Recklinghausen's disease) has been found to be associated with parathyroid enlargements. In experimental rickets and other conditions the enlargement of these glands seems to be secondary to the deficiency. However, accumulated clinical evidence favors hyperparathyroidism as the cause of von Recklinghausen's disease.² We have attempted to furnish experimental evidence for this view. The relative tolerance of guinea pigs to parathormone enabled us to induce in this animal a condition of severe but non-fatal chronic hyperparathyroidism, leading to *ostitis fibrosa* (3, 4).

We proceeded on the working assumption that while hypercalcemia is one indication of parathormone action, its reported absence in guinea pigs was not a proof of immunity to parathormone, for parathormone might cause the mobilization and excretion of calcium, as well as produce its other associated effects, without necessarily raising the serum calcium. Single injections of large doses (20 units per 100 gm.) of parathormone resulted not only in hypercalcemia but also in hyperphosphatemia, which were most pronounced in young guinea pigs fasted for 60 hours; severe and extensive bone resorption, with injury of the bone marrow, occurred only in young guinea pigs. Prolonged treatment with parathormone resulted in varying degrees of bone

¹ A preliminary report of this work has been published (1). The bone changes were reported in a companion paper (2), to which reference may also be made for protocols and for details which have been omitted from the present paper in order to avoid unnecessary duplication.

² Barr and Bulger recently reviewed the literature of this subject in the *Am. J. Med. Sci.*, 1930, 179, 449.

when overdosage was suspected. Parathormone doses were sometimes increased in special interpolated tests. In the experiments that were thus interpolated (E-1, E-2, etc.) care was taken not to interfere with the principal object of producing and maintaining a state of chronic hyperparathyroidism.

Serum Calcium and Phosphorus in Chronic Hyperparathyroidism

In preliminary experiments (9) to establish the limits of safe—or highest non-fatal doses,—and effective—or the lowest doses with which bone resorption could be demonstrated—we found that daily parathormone injections of 1 unit per kg. at no time resulted in overdosage or hypercalcemia at the 18 hour interval, even on a liberal calcium diet; slight bone resorption indicated a negative mineral balance. On a low calcium intake an initial dose of 2 units per kg. could be employed, which could be raised gradually to 6 units per kg. without symptoms of overdosage; bone resorption and fibrosis were definite. An initial dose of 4 units per kg. produced hypercalcemia and overdosage both on low and liberal calcium intakes, presumably due to an abundant store of readily available calcium in the tissues.

The experience gained in the preliminary tests enabled us to avoid fatal overdosage and to maintain a group of puppies in a condition of chronic hyperparathyroidism on varying calcium intakes.

Liberal Calcium Intake.—Puppy 7 (initial weight 1.3 kg.) received an average calcium supplement of 0.65 gm. daily and during most of the period of treatment (106 days) received 2 units of parathormone per kg. daily. (The details are given in Table I.)

Daily injection of 1.5 to 4 units per kg. was found at first to produce symptoms of overdosage, although hypercalcemia was absent (Periods 1, 2 and 3). Doses of about 5 and 4 units per kg. (Periods 5 and 7, respectively) resulted in more pronounced symptoms, with hypercalcemia, while hypercalcemia had been absent or slight and no symptoms of overdosage had appeared in the intervening Period 6, on 2.5 units per kg. The animal was therefore continued on about 2 units per kg. Its condition remained good.

The puppy was sensitive to parathormone throughout: On the 67th day of the experiment (Period E-1), on the 97th day (Period E-2c) and the 105th day (Period E-3b) single doses caused the serum calcium to rise about 1 mg. per unit per kg.

It is noteworthy that in experimental Periods E-2c and E-3b the rise of serum calcium after a single dose of parathormone was accompanied or followed by a rise of serum phosphorus (see Discussion, page 601).

The typical changes of *ostitis fibrosa* were found at autopsy.

injections, which are different in chronic hyperparathyroidism from the typical responses of normal dogs, and tested in dogs the assumption of immunity that has been advanced to explain the absence of hypercalcemia in man after repeated parathormone injections (10, 11). The bone changes were studied by histological methods and the findings are given here very briefly, as an indication of a negative mineral balance under the various conditions, in presence as well as in absence of hypercalcemia. They have been reported in detail in another paper (2). A chemical study of the effects of chronic hyperparathyroidism upon bone composition will be published later.

Experimental Methods

The puppies, 6 to 9 weeks old, and weighing 1 to 2 kg. at the beginning of the experiments, were of the same series as those reported in the companion paper (2), where brief diet notes were given. We have found our diet—lean meat supplemented with about 10 per cent canned tomato and 1 cc. cod liver oil—adequate for rapid growth over long periods, even when no calcium supplement was given, although in the latter case osteoporosis developed in controls not receiving parathormone (12).

Calcium Supplement.—In order to ascertain the effects of calcium intake, the basal (low calcium) diet was given without calcium supplement, and with small, adequate and liberal calcium supplements (10 per cent calcium lactate solution by stomach tube, or bone meal and calcium lactate mixed with a small portion of the meat mixture). Calcium supplement was sometimes omitted for 1 day before a special interpolated test (see page 594), in order to avoid possible effects of ingested calcium. An interval of almost 48 hours was thus allowed, although the serum calcium rise after the ingestion of calcium disappeared in parathormone treated animals within a few hours (12).

Parathormone Injections.—Parathormone (Lilly) was injected subcutaneously, usually at about 4 p.m., unless contraindicated. Anorexia, even in the absence of hypercalcemia, was a generally reliable indication of overdosage, and of the imminence of more urgent symptoms.

Blood Analysis.—The food was removed about 18 hours before the blood was drawn for analysis. Blood was drawn from the jugular vein. Serum calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method. The Benedict-Preis method was employed in the serum phosphorus analyses.

We adopted intervals of about 18 hours after injections of parathormone as most suitable for the determination of overdosage effects on the serum calcium and phosphorus (see Discussion, page 601). To study the effects of a single dose in detail we employed shorter intervals.

Recovery Periods and Special Tests.—Parathormone was sometimes discontinued or reduced during recovery periods (indicated in the tables as R-1, R-2, etc.)

Liberal Calcium Supplement after a Control Period on a Low Calcium Supplement.—Another puppy (No. 6, initial weight 1.1 kg.) was placed for 6 weeks on an average daily calcium supplement of 0.125 gm. and on daily doses of parathormone increasing from 2 to 6 units per kg. As usual, hypercalcemia associated with symptoms of overdosage was found early in the treatment. The serum calcium was, however, normal (10.6 mg. per 100 cc.) during the last week of the period, on 6 units per kg. daily; the serum phosphorus was 7.5 mg. per 100 cc.; the puppy had been eating well and gaining weight.

The daily calcium supplement was then increased to 1.2 gm., the dose of parathormone remaining the same. Pronounced hypercalcemia (15.3 mg. of calcium per 100 cc.) followed. The calcium supplement was reduced to 0.65 mg. daily and the parathormone to 4 units per kg. Hypercalcemia (19.0 and 18.5 mg. of calcium per 100 cc.), was followed later by evidence of compensation (12.6 and 11.3 mg.). The animal, however, declined with loss of appetite and other symptoms of overdosage. Bone and soft tissue changes due to terminal acute hyperparathyroidism were found, superimposed upon some bone resorption with marrow fibrosis due to chronic hyperparathyroidism.

Thus, even after a long period of treatment, doses well tolerated on a lower calcium intake proved fatal on a liberal calcium intake.

Calcium Deficient Diet.—Puppy 8 (initial weight 1.1 kg.) received no calcium supplement except during a short period of recovery, when calcium administration seemed necessary to prevent the animal's decline and death (R-2), and during a final experimental period (E-6). In this animal some of the characteristic phenomena of chronic hyperparathyroidism were observed most clearly and the data (given in Table II) are therefore discussed in greater detail.

The low calcium diet apparently enabled this animal to tolerate relatively large doses of parathormone (up to 6 units per kg. in Period 4) without hypercalcemia or symptoms of overdosage. Prolonged parathormone administration resulted eventually in a striking hypocalcemia, which was associated with hyperphosphatemia, which was particularly pronounced when the animal developed tetany or rigor (Periods 6, 7 and R-2). It is noteworthy that when the puppy went into tetany its serum calcium was considerably higher and its serum phosphorus lower than in tetania parathyreopriva (about 8 and 12 mg. per 100 cc., respectively, as compared with about 6 and 15 mg.) (12). Calcium lactate by stomach tube gave prompt relief from tetany; as a result of continued calcium treatment, the post-absorptive serum calcium rose from about 8 mg. per 100 cc. to over 9 mg. at the end of Period R-2 and during Period E-1. To save the life of the animal, parathormone was discontinued for 44 days (Periods 8 and 9); the animal gained weight for about 5 weeks; the serum calcium remained consistently low and the serum phosphorus rose. After parathormone administration had been resumed (Period 10) the serum calcium remained low when not under the influence of the last dose of parathormone. The serum phosphorus was definitely lower during the late part of the treatment.

TABLE 1⁴*Course of Chronic Hyperparathyroidism on a Liberal Calcium Intake*

Period No.	Duration	Weight (end of period)	Daily treatment			Serum analyses				Remarks
			Average food intake	Calcium supplement	Parathormone	Day	Hrs. after injection	Calcium	Phosphorus	
	days	kg.	gm.	gm.	units					
1	7	1.5	110	1.3	2					Vomited repeatedly
2	17	1.8	150	.60	4	4	18	11.9		" "
3	4	2.0	200	.60	8	3	18	11.3		" "
4	2	2.0	250	.60	12	1	20	Lost		No food taken on 2nd day
R-1	4	2.0	110	.60	0	3	18	11.4		Recovery period
5	4	2.1	240	.60	10	4	18	15.6	6.5	Anorexia on last day
R-2	2		210	.60	0					Recovery period
6	21	3.5	360	.60	8	1	18	12.2	8.9	Condition good
						4	18	12.6		
						12	24	13.7	9.0	
						19	21	11.1	8.6	
7	3	3.2	120	.20	14	1	20	14.5	7.8	Appetite failed rapidly
R-3	1		140		0					Recovery period
E-1	1			0	12		22	17.1	5.2	Test of effects of a single dose
R-4	2	3.0	0		0					Recovery period. Refused food, but active
8	7	3.3	300	.65	6	5	21	11.5	7.0	Condition good henceforth
9	18	4.0	370	.65	8	5	18	11.2	8.5	
						12	18	11.0	8.0	
E-2a	1		320	.65	0					
2b	1		0	0	0					
2c	1			0	8		Init.	10.8	8.7	Test of effects of a single dose
							3	12.8	8.5	of parathormone
							6	12.6	8.9	
							9	12.0	9.3	
							12	11.2	9.3	
							24	9.8	8.5	
10	6	4.4	465	.65	8					
E-3a	1		0	0	0					
3b	1			0	8		Init.	12.0	6.4	Test of effects of a single dose
							3	13.4	7.2	of parathormone
							24	12.0	6.5	

Serum calcium and phosphorus are stated in mg. per 100 cc.

⁴ For convenience of presentation periods of treatment with a given dose are distinguished by number in the tables from periods of recovery from overdosage (R-1, R-2, etc.) and from experimental periods (E-1, E-2, etc.) interpreted to elucidate certain special problems.

TABLE II⁴—*Concluded*

Period No.	Duration	Weight (end of period)	Daily treatment			Serum analyses				Remarks
			Average food intake	Calcium supplement	Parathormone	Day	Hrs. after injection	Calcium	Phosphorus	
	<i>days</i>	<i>kg.</i>	<i>gm.</i>	<i>gm.</i>	<i>units</i>					
11	20	3.3	245	0	8	8	13	8.0	6.7	Note higher calcium value at the 8 hour interval
						12	8	9.2	6.3	
						20	20	8.0	7.3	
E-3a	1		300	0						Test of effects of a single dose; rigid when handled
3b	1			0	16		Init.	7.9	7.8	
							9	8.6	8.5	
12	25	3.4	230	0	8	5	22	7.6	8.6	Progressively increasing weakness and skeletal deformities
						19	22	7.5	8.0	
E-4a	1		110	0	0					Test of effects of a single dose of parathormone
4b	1			0	30		Init.	7.7	7.2	
							9	8.6	8.9	
13	2		0	0	8					Animal refused to eat
E-5	1			0	70		20	10.7	7.2	Test of a single large dose
E-6	7	2.9	90	.10	20	3	20	9.6	6.2	Bloody diarrhea
						6	20	7.8	6.4	

The puppy remained sensitive to single doses of parathormone even in the late stages of chronic hyperparathyroidism, as shown by its serum calcium about 9 hours after the injection. (Periods E-1b, 62nd day; E-2, 95th day; 11, 121st day; E-3b, 130th day; E-4a, 157th day of the treatment). Plateau effects were obtained with very large doses (E-5 and 3rd day of Period E-6). The serum phosphorus also rose after single doses of parathormone (E-3b and E-4b).

Thus, even toward the end of the experiment immunity to parathormone had not been established, although, due to the reduction of the readily available calcium reserves, the conditions were obviously not favorable to a demonstration of a marked calcium rise from the usually hypocalcemic level, except with very large doses. The serum calcium rise (per 100 cc.) was at first about 0.5 mg. per unit per kg., decreasing later.

The typical changes of *ostitis fibrosa* were produced in this animal in the severest form. The effect of chronic hyperparathyroidism was thus enhanced by calcium deprivation, but was distinct from that of calcium deprivation as such—osteoporosis, which is easily distinguishable in the gross examination of the skeleton.

Calcium Deficient Diet Preceded by an Adequate Calcium Intake.—In another puppy (No. 10, initial weight 1.1 kg.), a period of about 60 days on a calcium

TABLE II⁴*Course of Chronic Hyperparathyroidism on a Calcium Deficient Diet*

Period No.	Duration	Weight (end of period)	Daily treatment				Serum analyses				Remarks
			Average food intake	Calcium supplement	Parathormone	Day	Hrs. after infection	Calcium	Phosphorus		
	days	kg.	gm.	gm.	units						
1	7	1.2	1100	0	2					Condition good	
2	17	1.6	1300	0	4	4	18	13.0		" "	
3	5	1.7	1800	0	6	3	18	11.4		" "	
4	5	2.0	2200	0	12	4	18	10.9		" "	
5	5	2.1	3000	0	16					Vomited on last day	
R-1	1		800	0	0					Recovery period	
6	8	2.4	2400	0	6	1	18	9.6	9.4		
						8	18	8.1		Sensitive to handling	
7	3	2.4	3000	0	10	3		8.1	9.4	Tetanic seizures, frequent at	
R-2	9	2.6	2400	.60	0	1		7.8	10.7	first, with respiratory failure,	
						2		9.9	7.1	spasmodic defecation	
						3		8.7	11.0	and urination; marked	
						4		8.9	9.4	weakness. Gradual improvement	
						7		9.4	9.1	in Period R-2	
E-1a	1		0	0	0			9.2		Controls on values in Period	
								9.0		E-1b below, at the same	
								8.7		hours	
								8.7			
E-1b	1		0	10		Init.		8.7		Test of effects of a single dose	
						3		8.7		of parathormone	
						9		11.0			
						22		9.3			
8	32	3.3	3400	0	0	3		9.9		Condition relatively good at	
						6		8.2	7.5	first, but getting weaker toward	
						9		8.4	8.5	the end of the period,	
						14		9.2	8.1	and more sensitive to handling	
						17		8.5	9.0		
						24		8.5	10.0		
E-2	1		0	6		Init.		8.0	10.0	Test of effects of a single dose	
						3		9.2	12.4	of parathormone; tetanic	
						6		8.4	10.0	seizure when handled at the	
						9		8.8	10.5	3 hour interval	
						12		8.2	10.1		
						24		7.8	10.1		
9	11	3.1	2200	0	7			7.8			
10	2	3.1	2150	0	3						

Serum calcium and phosphorus are stated in mg. per 100 cc.

TABLE III⁴*Effect of Changes of Calcium Intake upon the Course of Chronic Hyperparathyroidism*

Period No.	Duration	Weight (end of period)	Daily treatment			Serum analyses				Remarks
			Average food intake	Calcium supplement	Parathormone	Day	Hrs. after injection	Calcium	Phosphorus	
	days	kg.	gm.	gm.	units					
1	6	2.0	200	.20	4					
2	4	2.0	160	.20	8	3	18	16.4		Loss of appetite
R-1	6	2.1	160	.20	0	5	18	11.3		Recovery period
3	4	2.3	255	.20	6	4	20	12.4	6.9	Overdosage suspected
R-2	2		245	.20	0					Condition good
4	2	2.1	130	.20	8	1	20	11.4	7.3	Vomited on 2nd day
R-3	2	2.1	125	1.3	0	2	68	11.0		Recovery period
5	17	3.0	265	.65	8	8	24	15.0	6.5	Appetite better; condition
						9	21	15.5	6.3	good
						15	24	10.9	6.1	
6	7	2.8	180	.65	12	1	24	12.1	7.0	Appetite decreased toward the
						5	20	11.1	7.0	end of the period
R-4	8	3.0	240	.65	6	5	21	11.0	5.8	Dose reduced; condition
										better
7	25	3.8	380	.65	8	4	24	10.7	5.6	Condition good
						11	18	10.9	6.1	
E-1a	1		0		0					
1b	2		0		8	1	Init.	12.4	5.4	Test of effect of single dose at
							3	13.8	5.9	the end of régime of liberal
							25	11.8	4.8	calcium intake
8	24	4.0	350	0	8	12	12	10.0	6.5	Condition good
						16	8	9.6	6.6	Note decreased parathormone
						24	20	9.6	5.1	effect at early intervals
E-2a	1		300	0	0					
2b	1		0		16		Init.	9.6	6.4	Test of effect of a single dose
							9	12.2	6.6	
9	25	5.1	450	0	8	19	20	8.5	7.4	Note hypocalcemia
E-3a	1		510	0	0					
3b	1		0		30		Init.	8.7	7.9	Test of single dose. Note
							9	9.3	7.9	continuing hypocalcemia
10	3	5.2	515	0	10					
E-4	25	5.4	370	.10	20	3	20	9.9	6.9	Gradual decrease of appetite;
						6	20	10.1	6.8	refused food on last day of
						10	18	10.8	6.9	the period. Note gradual
						17	18	10.7	6.2	decline of serum phos-
						24	24	10.7	6.1	phorus

Serum calcium and phosphorus are stated in mg. per 100 cc.

deficient diet was preceded by a period of about 100 days on an average daily calcium supplement of 0.2 gm. Hypercalcemia and anorexia were marked early in the treatment on a dose of parathormone of 1.5 to 4 units per kg., and were still observed as late as the 67th day of the treatment; hyperphosphatemia (9 to 10 mg. per 100 cc.) in absence of hypercalcemia was frequent, and hypotonia appeared. The dose was reduced to 2 units per kg., and the animal gained weight, showing a normal serum calcium but a high serum phosphorus (8 to 9 mg. per 100 cc.).

After the elimination of the calcium supplement, increasing doses (up to 4 units per kg.) were required to produce an equal serum calcium elevation, which was consistent although slight, and was associated with a characteristic serum phosphorus rise (see Discussion, page 601). The serum phosphorus rose from an average initial value of 7.0 mg. per 100 cc. to an average of 8.0 mg. within 3 to 9 hours after the injection.

During a terminal 25 day period on 20 units of parathormone daily (4 units per kg.) a consistent decline of serum phosphorus occurred from an average of about 7 mg. per 100 cc. at the beginning of the period to about 6 mg. at its end. Hypercalcemia and urgent symptoms of overdosage were absent. On the other hand, hypocalcemia was prevented by an average supplement of 0.1 gm. of calcium per day.

The typical lesions of *ostitis fibrosa* were produced, but not in as severe form as in Puppy 8.

Variations of Calcium Intake.—Puppy 9, initial weight 2.3 kg., received an initial daily calcium supplement of 0.2 gm. for 24 days, 0.65 gm. for 57 days, no supplement for 59 days and 0.1 gm. for 25 days. (The details are given in Table III.)

On a supplement of 0.2 gm., early hypercalcemia appeared as usual, but soon disappeared, to reappear (Period 5) when the supplement was increased to 0.65 gm., the parathormone dose remaining constant (4 units per kg.). But even on this liberal calcium intake hypercalcemia tended to disappear (Periods 5 to 7 inclusive). The puppy gained weight during Period 5, on about 3 units per kg.; when parathormone was increased to 4 units per kg. (Period 6) symptoms of overdosage appeared, without hypercalcemia, but with a slight increase in serum phosphorus (as compared with the figures in Periods 5 and R-4); after a decrease of the dose to 2 units per kg. (R-4 and 7) the animal gained weight and was otherwise in good condition.

2 and 29 days after the elimination of the calcium supplement, serum calcium showed definite effects of a single dose (Periods E-1b and E-2b), but very slight effects were shown 25 days later (Period E-3b). A tendency to hypocalcemia had already appeared, as in Puppy 8. Severe hypocalcemia, with associated extreme effects, as observed in Dog 8, was prevented by a small calcium supplement. As in Dog 10, the final long period of relative overdosage resulted in a gradual decline of serum phosphorus.

The typical changes of *ostitis fibrosa* were produced, not in as pronounced form as in Dog 8, and substantially identical with those in Dog 10.

phatemia found in the clinical cases of *ostitis fibrosa cystica* (von Recklinghausen's disease). This difference may be due to the age of the animal, as well as to the method of treatment which frequently approached the limit of the dog's tolerance. There are, of course, many differences between the experimental procedure and the spontaneous clinical process. Age and species differences, as well as the duration of the state of hyperparathyroidism are among the factors that may affect the various associated phenomena.

We have found, for instance, ready bone resorption after single and repeated doses of parathormone in young guinea pigs, but not in the adult (2, 4). Hirsch (13) suggested that the clinical condition begins frequently in childhood and has a long course before it becomes clinically obvious.

The influence of the species factor is illustrated by a comparison with our results in chronic hyperparathyroidism of guinea pigs (4). As a result of previous treatment with smaller doses, a modified response to parathormone appeared both in the guinea pig and in the dog, and toxic symptoms tended to disappear. In that stage, hypercalcemia was observed in the guinea pig without hyperphosphatemia, while in the dog, hyperphosphatemia was present, while hypercalcemia was frequently absent, particularly on a limited calcium intake.

The common symptoms of chronic hyperparathyroidism were most severe in Puppy 8, on a calcium deficient diet, but were present, although in not as severe form, in the other puppies. Among these the most important were general hypotonia, muscular weakness and bone deformities with frequent fractures—also characteristic findings in clinical cases of hyperparathyroidism.

The essential bone lesions of *ostitis fibrosa*, which were generalized (involving all the skeletal bones studied—skull, jaw bone, long bones, ribs), were most severe on low calcium diets, but were distinct from the effects of low calcium diets as such. While calcium deficiency *per se* is quite compatible with rapid growth, parathormone administration retarded bone formation and growth in a very striking manner (2). Extensive bone and marrow fibrosis, with the production of osteoid tissue and cysts, was the end result of the drastic combined effects of calcium deficiency and chronic hyperparathyroidism. In dietary

DISCUSSION

In chronic hyperparathyroidism the curves seem to be quite different from those observed in normal dogs after single injections of parathormone (11); the effect of a single moderate dose reaches its maximum before the 12th hour, and disappears within 18 hours; after large doses hypercalcemia may persist beyond that interval (plateau effect); when hypercalcemia is absent, the serum phosphorus will frequently be raised at the same late interval after an excessive dose. We therefore consider an interval of at least 18 hours after the injection of parathormone as suitable for the detection of overdosage in experimental chronic hyperparathyroidism. Earlier intervals may be employed for a more detailed study of responses to moderate as well as large doses.

While the influence of the calcium intake on the serum calcium response to parathormone which we observed was perhaps to be assumed *a priori*, the relative unimportance of this influence in young animals at the beginning of the parathormone treatment is of particular interest, indicating the influence of readily available reserves on the serum calcium after parathormone injections. The tolerance to parathormone that Morgan and Garrison (8) found in animals on a vitamin free diet is analogous in some respects to that found on a calcium poor diet later in the treatment.

While in normal dogs the serum phosphorus is lowered by a single dose of parathormone, the serum phosphorus rose in puppies suffering from chronic hyperparathyroidism at early intervals (9 hours and earlier) after a single moderate injection. Sometimes the increase of serum phosphorus was the earliest response and frequently the only response to a single parathormone injection in these dogs, when the serum calcium rose slightly, if at all. Thus, after long periods of parathormone treatment absence of hypercalcemia points not to immunity but to a modification of the response to parathormone. We are continuing the study of this phase of the subject.

The similarity between the association of low serum calcium and high serum phosphorus in chronic hyperparathyroidism on low calcium diets and that observed in renal rickets suggests further investigation.

The hyperphosphatemia distinguishing experimental chronic hyperparathyroidism in young dogs was also in contrast to the hyperphos-

nent on low calcium intakes, which permitted the use of large doses of parathormone without fatal hypercalcemia and without symptoms of overdosage.

7. Clinical and experimental hyperparathyroidism are compared and discussed.

The courtesy of Eli Lilly and Company, who supplied the parathormone used in these experiments, is acknowledged.

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calcium deficiency a certain economy of calcium is still possible. With chronic hyperparathyroidism added, it was rendered more difficult.

SUMMARY

1. On a low calcium intake hypercalcemia tended to disappear in chronic hyperparathyroidism on a given dose of parathormone (as large as 6 units per kg.), apparently due to the reduction of a readily available calcium reserve. An increase of either the calcium intake or of the daily dose of parathormone caused a rise of serum calcium and symptoms of overdosage.

2. Hypocalcemia developed in chronic hyperparathyroidism in young puppies on a low calcium diet. Tetany occurred at a calcium level which was higher and a phosphorus level which was lower than in tetania parathyreopriva of young puppies. About 0.1 gm. of calcium daily was apparently sufficient to maintain the serum calcium at a normal level.

3. The serum phosphorus in chronic hyperparathyroidism in young puppies continued at or rose above the high level normal for young animals. Toward the end of long periods of treatment on large parathormone doses (about 5 units per kg.) serum phosphorus approached normal levels, pronounced hypercalcemia was absent but hypotonia and other symptoms of hyperparathyroidism were present.

4. A single dose of parathormone caused early in the treatment and on liberal calcium intakes a more marked relative rise of serum calcium than in normal adult dogs, confirming previous observations (5, 8). Later in the treatment and on low calcium intakes this effect was greatly reduced. Serum phosphorus rose after a single injection of parathormone, even when the effect on the serum calcium was slight or absent.

5. The continued effect of parathormone on serum calcium after prolonged periods of treatment, and the modified response of the serum phosphorus indicate tolerance due to some compensation, rather than immunity.

6. The bone lesions, presenting the essential features of *ostitis fibrosa cystica* (von Recklinghausen's disease) in varying degrees of severity, depending on the relation of the parathormone dose to the calcium intake and to the duration of the treatment, were most promi-

greater tendency to combine with reagin, explaining the greater sensitivity of cholesterolized antigen in the Wassermann test.

It was shown that cholesterol, if it were sufficiently soluble, could be used in as high a concentration as 6 per cent and yet continue to increase the sensitivity of the antigen-lipoids by this mechanism (Eagle, 1930, *b*, Fig. 3). The almost universal use of only 0.2 per cent cholesterol or less has neither theoretical nor practical justification. As will be shown in a later paper, providing that technical error can be excluded, the supposed danger of producing biologically false positive tests if higher cholesterol concentrations are used does not exist. However, since the limit of its alcohol solubility is only about 0.6 per cent, even saturating the antigen with cholesterol does not exhaust the possibilities of sensitization.

Because the coarse dispersion caused by cholesterol is the basis of its effect and is due solely to certain physical properties, it was predicted (1) that many other water-insoluble, alcohol-soluble substances would have the same effect; and (2) that if these substances were sufficiently soluble in alcohol, they might be used to replace or supplement cholesterol, with a corresponding increase in the sensitivity of the antigen. This prediction has been realized in the discovery that a group of chemically unrelated substances have fortifying properties similar to those of cholesterol. Their use has made possible the preparation of an antigen which is much more sensitive than any now available.

The Preparation of a Concentrated Antigen

If dry powdered beef heart is extracted with alcohol, one obtains a deep yellow liquid which is highly anticomplementary, containing, along with the active lipoid, inactive and anticomplementary fatty acids, fats, soaps, etc. It has therefore become customary either (1) to precede the alcohol extraction by repeated ether extractions, which remove practically all the interfering anticomplementary factors, or (2) to precipitate the complex alcoholic extract with acetone, in which the active lipoids are insoluble, while most of the undesirable substances remain in solution. As shown by the following experiments, however, both these methods of purification involve the loss of fully $\frac{1}{2}$ to $\frac{3}{4}$ of the active lipoid (Protocol 1 and Table I).

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IV. A MORE SENSITIVE ANTIGEN FOR USE IN THE WASSERMANN REACTION*

BY HARRY EAGLE, M.D.†

(From the Syphilis Division of the Department of Medicine, Johns Hopkins Medical School, Baltimore)

(Received for publication, February 7, 1931)

The lipoids used as antigen in the Wassermann test are generally obtained by alcoholic extraction of beef heart muscle. Because there is no uniform method of preparation, the efficiency of antigens from different laboratories or those made at different times in the same laboratory show wide variations.

No matter how prepared, however, there are two important factors which determine the sensitivity of the extract obtained. The higher the percentage content of active lipoid in an antigen, the more sensitive is the Wassermann test, *i.e.*, the less "reagin" need be circulating in the blood in order to give a positive reaction. The second and by far the more important factor is the amount of cholesterol used to fortify ("sensitize") the antigen.

Cholesterol, although in itself inert, increases the sensitivity of the alcoholic tissue extract enormously. The phenomenon has been explained in detail in a previous paper of this series (Eagle, 1930, *b*). An alcoholic solution of the antigen-lipoids alone, when dropped into water, forms a barely opalescent colloidal suspension in which most of the particles are too small to be seen microscopically. Cholesterol causes a coarser dispersion; relatively few particles of much larger average size are obtained, each consisting of an inert core of cholesterol surrounded by a layer of antigen. Such large particles have a much

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and washings were then concentrated on the steam bath to a volume corresponding to 2.5 to 3 cc. per gram of powder originally used.

The Fortification of the Lipoid Extract

A. Substitutes for Cholesterol.—As suggested in a preceding paper, 50 water-insoluble, alcohol-soluble substances were tested for their sensitizing properties. Of these ten were discarded because their alcoholic solutions, when dropped into NaCl N/7 are colloiddally dispersed, and therefore do not have the desired effect. Eight could not

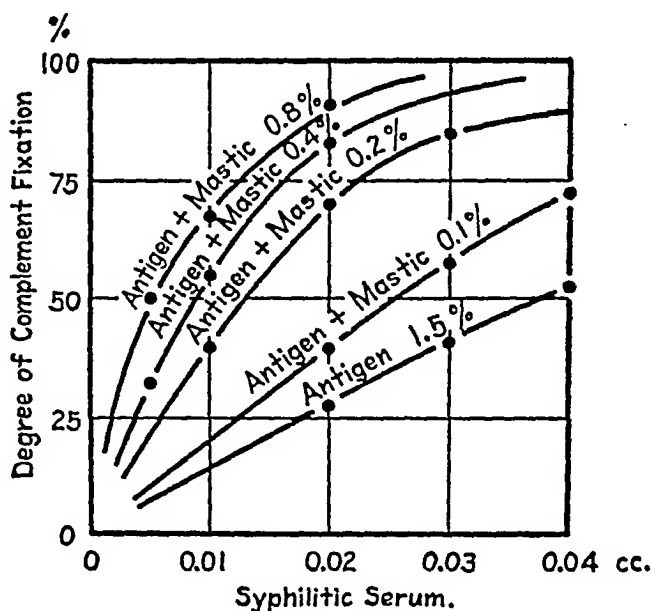


FIG. 1. The sensitizing action of mastic upon an alcoholic extract of beef heart.

be used because the addition of antigen does not prevent their flocculation when dropped into saline, either because the antigen is not adsorbed or because the substances in question have so pronounced a tendency to aggregate that an incomplete film of antigen does not sufficiently protect. Some very active sensitizing agents fall into this latter group (palmitic acid, β -naphthyl acetate, etc.), but the presence of coarse aggregates precludes their use in the Wassermann reaction.

Thirty-two substances remained to be tested. All flocculate spontaneously when the alcoholic solution is dropped into NaCl N/7;

Protocol 1

(a) A weighed quantity of antigen was extracted four times with three volumes of ether, each extraction lasting 10 minutes. The combined filtrates were evaporated in the steam bath, and redissolved in 95 per cent alcohol (5 cc. per gram). The alcohol-insoluble residue was discarded. The dried residue remaining after the ether extractions was shaken with 95 per cent alcohol (5 cc. per gram), and filtered after 3 days of room temperature. The two alcoholic solutions, (1) of ether-soluble and (2) of ether-insoluble, alcohol-soluble lipoids were compared for antigenic activity by diluting with 160 volumes of saline and determining the minimal quantity necessary to give a fixation with 0.1 cc. of a strongly positive syphilitic serum (Table I-a). The figures in the body of the table indicate the per cent of complement fixation, determined by a method described elsewhere (Eagle, 1929; Eagle, 1930, a).

TABLE I

Per cent of complement fixation by (a) ether-soluble and ether-insoluble and (b) alcohol-soluble and acetone-insoluble beef heart lipoids with syphilitic serum. (See Protocol 1.)

Figures in body of table represent per cent of complement fixation.

1:160 dilution, cc.....		0.4	0.2	0.1	0.05	0.025	Total antigenic activity
							<i>per cent</i>
a {	ether-soluble lipoids.....	>90	>90	>90	75	40	60
	ether-insoluble lipoids.....	>90	>90	85	55	30	40
b {	alcohol-soluble lipoids.....	>90	>90	>90	>90	65	100
	acetone-insoluble lipoids.....	>90	>90	65	40	20	25

(b) A weighed quantity of antigen was extracted for 3 days with five volumes of 95 per cent alcohol. Part of the filtrate was concentrated to one-fifth its volume, and precipitated with twenty volumes of acetone. The precipitate was redissolved in the original volume of alcohol, and the loss in antigenic activity determined as outlined in Table I-b.

One short extraction (15 minutes) with five volumes of ether was found to suffice, removing more than 70 per cent of the undesirable substances and less than 30 per cent of the active lipoid. The dry residue was then extracted for 3 days with 95 per cent alcohol. After filtration, a certain amount (about $\frac{1}{4}$) of the extract remained on the moist powder. This was recovered by washing with two portions of alcohol, each $\frac{1}{2}$ the volume originally used. The combined filtrate:

on the substance, but also upon the concentration of the antigen lipoids and upon the amount of saline used in diluting. Although diphenyl has the peculiarity of causing a slow fine aggregation even in very small concentrations, its use is not thereby precluded; the antigen-diphenyl particles are not large enough to sediment, and the suspension remains stable and uniform for hours. (3) Finally, when antigen is diluted with saline for use in the Wassermann test, it forms a suspension the opacity (turbidity) of which is determined almost entirely by the amount of sensitizer used. (Eagle, 1930, *b*, Fig. 1.) Too strongly

TABLE II

Sensitizing Efficiency of the Various Alcohol-Soluble, Water-Insoluble Substances Tested

Slight	Fair	Very good
Gum guaiac	Gum balsam	Cholesterol
Gum sandarac	Gum copal	Sitosterol
Salol	Gum mastic	Gum thus
Gum shellac	Gum elemi	Safrole
Resin	Tolu balsam	Diphenyl
Benzoin	Benzophenone	Dimethyl naphthylamine
Terpineol	Aurin	Methyl stearate
Benzil		Methyl palmitate
<i>p</i> -iodobenzene		
Ethyl- <i>m</i> -nitrobenzoate		
β -Chloronaphthalene		
Triphenyl phosphine		
α -naphthonitrile		
<i>p</i> -nitrobromobenzene		
Ethyl palmitate		
<i>p</i> -Bromotoluene		

sensitized an antigen forms so opaque a dilution as to obscure the results of hemolysis.

The best of these newly discovered sensitizers is sitosterol, a sterol obtained from the non-saponifiable fraction of wheat germ. Its use in conjunction with cholesterol has been found to provide ample sensitization. None of the other substances is quite as active, and some, notably gum thus and dimethylnaphthylamine, cause a slow chemical change in the antigen preventing their use in high concentration.

but when they are dissolved in the fluid containing the antigen and the mixture is dropped into an excess of saline, they form a stable colloidal suspension. As shown by the greater turbidity and the enormously large number of microscopically visible particles, these substances all cause a coarsened dispersion of antigen. Since this is the mechanism of the cholesterol effect, all these substances should fortify the antigen. A single protocol is given in detail. (Protocol 2, Fig. 1.)

Protocol 2

A series of antigens were prepared as outlined below, each containing 1½ per cent of antigen lipoids, but varying quantities of gum mastic.

3 per cent antigen in alcohol, cc.....	1	1	1	1	1
2 per cent gum mastic in alcohol, cc.....	0	0.1	0.2	0.4	0.8
Alcohol, cc.....	1	0.9	0.8	0.6	0.2

A 1:40 dilution of each was used as antigen in a quantitative complement fixation test with a strongly positive syphilitic serum by a method described elsewhere (Eagle, 1930, *a*) The results are summarized in Fig. 1.

All but three of the substances tested had a qualitatively similar effect to that of gum mastic as just described, sensitizing antigen so that less reagin is necessary to give a positive Wassermann reaction. They are grouped in Table II in the order of their sensitizing efficiency.

Although cholesterol is the best sensitizer, at least five of the substances tested (sitosterol, gum thus, safrole, diphenyl, and dimethylnaphthylamine) approximate to it so closely (75 to 90 per cent) in this particular as to be of practical importance.

B. Optimum Quantity of Sensitizer to Be Added.—As shown in Fig. 1, the efficiency of an antigen increases almost indefinitely with the quantity of sensitizer; but there are three limiting factors to the amount which can be added. (1) The first is the anticomplementary action of the sensitizer, most marked with gum thus, and safrole, but relatively unimportant with cholesterol, sitosterol, diphenyl, or dimethylnaphthylamine. (2) The second is the fact that an excess of sensitizer causes visible flocculation when the antigen is diluted with saline. The exact value of this stability threshold depends, not only

appears to be one of the most sensitive of biological tests. A 1:10,000 dilution of the antigen sufficed to give 4+ fixation with a very strongly positive serum: *i.e.*, $\frac{1}{10,000} \times \frac{1}{5} \times \frac{2.5}{100} = \underline{0.0000005}$ gm. of beef heart lipid sufficed to detect syphilitic reagin.

A comparison with other antigens is best obtained, not by a laborious evaluation of the results in some thousands of cases, but by a quantitative comparison by the simple technique outlined in Protocol 3 and Table III, determining (a) the dilution in which a given syphilitic serum is still positive with a fixed quantity of antigen, and (b) the quantity of antigen necessary to give fixation with a constant quantity of syphilitic serum.

Protocol 3

(a) Decreasing quantities of an inactivated positive syphilitic serum were made up to a constant volume with normal inactivated serum, forming a series of sera of decreasing positivity.

	1	2	3	4	5	6	7
Syphilitic serum, cc.....	4	2	1	0.5	0.25	0.125	0.062
Normal serum, cc.....	0	2	3	3.5	3.75	3.775	3.9

0.2 cc. portions of each mixture were incubated with 0.2 cc. of each of the antigens listed in Table III, and 0.2 cc. of complement 1:10 for 16 hours at 8°C., after which the indicator system was added (sheep cells sensitized with four units of amboceptor). The figures in the body of Table III indicate the traditional degrees of fixation (4 = no hemolysis: 0 = complete hemolysis).

(b) 0.05 cc. of a strongly positive serum was incubated with complement and decreasing quantities of the various *antigens* listed in Table III, Section b.

It is evident that the antigen recommended is far more sensitive than any of the others tested.

It is characteristic of the confusion which surrounds the whole subject of the Wassermann reaction that the particular advantage of this antigen lies in just that fact which has been so often warned against as a dangerous procedure leading to false positives, namely, excessive sensitization. Because of this supposed danger, cholesterol is almost never used in concentration greater than 0.2 per cent.

Omitting the details of the preliminary orienting experiments, the following is the method finally decided upon:

To the basic extract prepared as described under the preparation of a concentrated antigen are added 0.8 per cent cholesterol, and 0.6 per cent sitosterol. These are dissolved by boiling, and the excess which crystallizes out upon cooling is redissolved just before using by immersing for a few minutes in a 56°C. bath. So sensitized, there is no flocculation in as high as 1:10 concentration when the antigen is diluted with saline. Although the colloidal suspension thus formed is milky white, the opacity disappears almost completely upon the addition of complement and serum, possibly owing to dispersion of fine aggregates by the serum protein acting as a protective peptizing colloid. Finally, as will be shown in the next section, despite its concentration, and despite the large quantities of added substances, the quantity of antigen used in the test is only a fraction of its anti-complementary titre, eliminating any possibility of a technical falsely positive result.

The Concentrated Cholesterol-Sitosterol-Antigen in Practice

Containing as it does some two to three times as much active antigenic lipoids, (2 to 3 per cent), and four to sixteen times as much sensitizing material (1.4 per cent) as most of the Wassermann antigens now in use, the antigen just described should *a priori* be many times as sensitive.

The quantity recommended for use in the test depends upon the quantities of the other reagents, as well as the time and temperature of incubation. With 0.2 cc. of 1:10 complement and 0.2 cc. of the serum to be tested (whole or diluted), one uses 0.2 cc. of the antigen, diluted 1:40 in saline for water bath incubation (37° for $\frac{1}{2}$ hour) and 1:120 for ice box fixation (8° for 16 to 24 hours). These quantities are $\frac{1}{4}$ and $\frac{1}{2}$ the anticomplementary unit of the antigen, respectively, a very wide margin of safety.

It has been pointed out elsewhere (Eagle, 1930, *b*) that the term antigen unit has no real significance; the minimal fixing quantity depends upon the titre of the syphilitic serum used in its titration. Qualitatively, however, the quantities of antigen recommended are 25 to 50 times this minimal fixing quantity, an index of the sensitivity of the test. Indeed, the Wassermann reaction, carried out with this antigen,

much more sensitive than any now available for use in the Wassermann reaction.

100 gm. of dry powdered beef heart muscle are extracted with 500 cc. ether for 15 minutes at 37°C. with shaking. After filtration *with suction*, the ether filtrate is discarded. The powder is then dried and extracted for 3 to 5 days with 500 cc. of 95 per cent alcohol, with intermittent shaking. The mixture is filtered, and the moist powder washed on the filter paper with two portions of alcohol, each 100 cc. The alcoholic filtrate and washings are combined and evaporated on the steam bath down to 250 to 300 cc. Cholesterol (0.8 per cent), and sitosterol (0.6 per cent) are then added, and dissolved at 65°-75°C. The excess sensitizer which crystallizes out upon cooling is dissolved just before using by immersing the antigen in a 56°C. bath for a few minutes. It is diluted by pouring the saline rapidly into the antigen. A 1:40 dilution is recommended for use in water bath fixation ($\frac{1}{2}$ hour at 37°C.), as well as for the short ice box fixation (8°, 4 hours) and a 1:120 dilution for use in the overnight ice box method (16 to 24 hours at 8°C.), as being well beyond its anticomplementary range; the anticomplementary quantities being 1:5 and 1:25 respectively.

There is reason to believe that this antigen possesses almost the maximum sensitivity obtainable. The method of preparation insures its being almost saturated with antigen-lipoids; and more sensitizer could not be added without increasing the turbidity of its dilution in saline to a point where it would interfere with the reading of hemolysis. Any further improvement must await the discovery of better sensitizers.

Preliminary experiments indicate that this new sensitizer, sitosterol, will find an immediate application, not only in the Wassermann reaction, but also in a more sensitive flocculation test to be described in a following paper.

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Yet this antigen, containing four to six times as much sensitizer (1.4 per cent) as antigens currently used, does not give any more false positives than the usual Wassermann antigen. The question of the falsely positive Wassermann will be considered in a forthcoming paper.

TABLE III
Comparative Sensitivity of Various Antigens

(a) Tested by minimal fixing quantity of a positive serum					
Antigen used	Dilution of positive serum in negative				
	1	1:2	1:4	1:8	1:16
1. Non-cholesterolized ether-insoluble, alcohol-soluble.....	4	2	0	0	0
2. Ether-insoluble, alcohol-soluble + 0.2 per cent cholesterol.....	4	4	2	0	0
3. Ether-insoluble, alcohol-soluble + 0.6 per cent cholesterol.....	4	4	4	0	0
4. Concentrated antigen, supersaturated with cholesterol and sitosterol.....	4	4	4	4	0
5. Acetone-insoluble: no cholesterol.....	4	0	0	0	0

(b) Tested by minimal fixing quantity of antigen

Antigen used	Dilution of antigen in saline							
	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
1	4	2	2	0	0	0	0	0
2	4	4	4	4	0	0	0	0
3	4	4	4	4	2	0	0	0
4	4	4	4	4	4	2	0	0
5	2	2	0	0	0	0	0	0

SUMMARY AND DISCUSSION

The discovery (1) that there are many substances with the sensitizing properties hitherto believed peculiar to cholesterol and its derivatives, and (2) that sensitizer can be added to antigen in very large quantities, many times those currently used, and yet continue to increase its complement-fixing efficiency with no danger of giving falsely positive tests has made possible the preparation of an antigen

such that approximately 80 per cent of the complement used must be destroyed in order to give a completely positive reaction (no visible hemolysis). In $\frac{1}{2}$ hour at $37^{\circ}\text{C}.$, antigen alone as used in the test causes no measurable destruction of complement: all 80 per cent must be actually fixed by the antigen-reagin interaction in order to give a positive test. On the other hand, in 16 to 24 hours at $8^{\circ}\text{C}.$, antigen alone may destroy as much as 30 per cent of the complement used: only 50 per cent need be fixed by the specific reaction with syphilitic serum (Fig. 2).

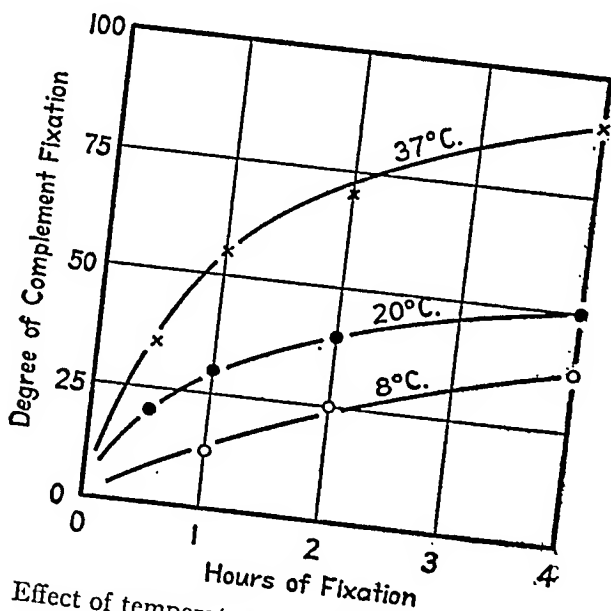


FIG. 1. Effect of temperature upon the velocity of fixation.

In the third place, complement decomposes spontaneously. The degree of this non-specific degeneration depends (1) upon the duration of the ice box incubation, and (2) upon the quality of the complement. It is clear that, like the destruction by antigen, this spontaneous change facilitates the production of a positive Wassermann (Fig. 2). Indeed, particularly poor complement may deteriorate so rapidly that in the overnight ice box test, every reaction appears to be positive, even though there has been no specific complement fixation *i.e.*, even though the serum is negative.

Although these three factors are the most important, particularly in the 16 to 18 hour ice box fixation as recommended by Kolmer, they

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V. THE CAUSE OF THE GREATER SENSITIVITY OF THE ICE BOX WASSERMANN; THE ZONE PHENOMENON IN COMPLEMENT FIXATION

By HARRY EAGLE, M.D.*

(From the Syphilis Division of the Department of Medicine, Johns Hopkins Medical School, Baltimore)

(Received for publication, February 7, 1931)

It has been known for many years (Jacobsthal, 1910, Guggenheimer, 1911; Coca and L'Esperance, 1913, etc.) that the sensitivity of the Wassermann reaction is greater if the first phase, the incubation of syphilitic serum, complement, and antigen, is carried out in the cold.

The explanation of this temperature effect is the subject of the present paper.

What is usually considered the only causal factor is the longer duration of fixation made possible by the lower temperature. As Dean (1916) showed, this more than compensates for the greater velocity of fixation at 37°C. (Protocol 1, Fig. 1). More complement is fixed in e.g. 12 hours at 8°C. than in $\frac{1}{2}$ to 1 hour at 37°C.

Protocol 1

To a constant amount of a strongly positive serum (0.005 cc.) were added complement and antigen, and the degree of fixation at 37°, room temperature, and 8°C. determined after varying intervals by a method described elsewhere. (Eagle, 1930,a). The results are summarized in Fig. 1.

In addition, there is the fact pointed out by Kolmer (1920) that the anticomplementary action of the antigen increases with time, facilitating by just so much the production of a positive Wassermann. Thus, by the technique used in this laboratory, the margin of safety is

* Aided by a grant from the Committee on Research in Syphilis, Inc.

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Sensitized cells were then added, the time for complete hemolysis noted, and the per cent of fixation determined by a method described elsewhere (Eagle, 1930 a). By the technique used, 80 per cent fixation corresponds to a routine 4 +, 65 per cent to a 1 +, and 65 to 80 per cent to intermediate degrees of hemolysis, i.e., 2 to 3 + fixation.

TABLE I
Titration of a Positive Serum by W.B. ($\frac{1}{2}$ Hour) and I.B. (3 Hours) Fixation

	0.4	0.2	0.1	0.05	0.0125	0.0125	0.062	0.031
Syphilitic serum, cc.....	0	0.2	0.3	0.35	0.375	0.4	0.4	0.4
NaCl N/7, cc.....	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Complement 1:10, cc.....	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Antigen 1:40, cc.....	>90	>90	>90	>90	>90	>90	80	45
Per cent fixation, I. B. 3 hrs..	>90	>90	>90	>90	>90	80	65	30
Per cent fixation, W. B. $\frac{1}{2}$ hr..	4+	4+	4+	4+	4+	4+	4+	0
Routine reading, I. B.....	4+	4+	4+	4+	4+	4+	1+	0
Routine reading, W. B.....								

TABLE II
Effect of Normal Serum upon Reagin Titre

	0.4	0.2	0.1	0.05	0.025	0.0125
Syphilitic serum, cc.....	0	0.2	0.3	0.35	0.375	0.4
Normal serum, cc.....	0.4	0.4	0.4	0.4	0.4	0.4
Complement 1:10, cc.....	0.4	0.4	0.4	0.4	0.4	0.4
Antigen 1:40, cc.....	>90	>90	60	35	10	0
Per cent fixation, W. B. $\frac{1}{2}$ hr.....	>90	>90	>90	70	40	25
Per cent fixation, I. B. 3 hrs.....	4+	4+	1+	0	0	0
Routine reading, W. B.....	4+	4+	4+	3+	0	0
Routine reading, I. B.....						

As seen in Table I, the serum in question gave a positive reaction (80 per cent fixation) by both methods, up to a 1:50 dilution (reagin titre of 50 in the water bath and up to 1:64 in the ice box).

If a similar titration is carried out on the same serum using negative inactivated serum as the diluent instead of NaCl N/7 one obtains the startlingly different results outlined in Table II.

The maximal dilution of serum which will give a positive reaction is no longer 1:64, but 1:7 by ice box, and 1:3 by water bath fixation;

are quite inadequate to explain all of the cases in which the results by the two methods differ. One repeatedly encounters sera which are negative by water bath fixation ($37^{\circ}\text{C}.$) no matter how long they are incubated; but which are clear cut positives after 4, 3, or even 2 hours at $8^{\circ}\text{C}.$

These are due to a fourth hitherto unsuspected factor which is also the cause of the so called zone phenomenon in complement fixation. It is described in the following section.

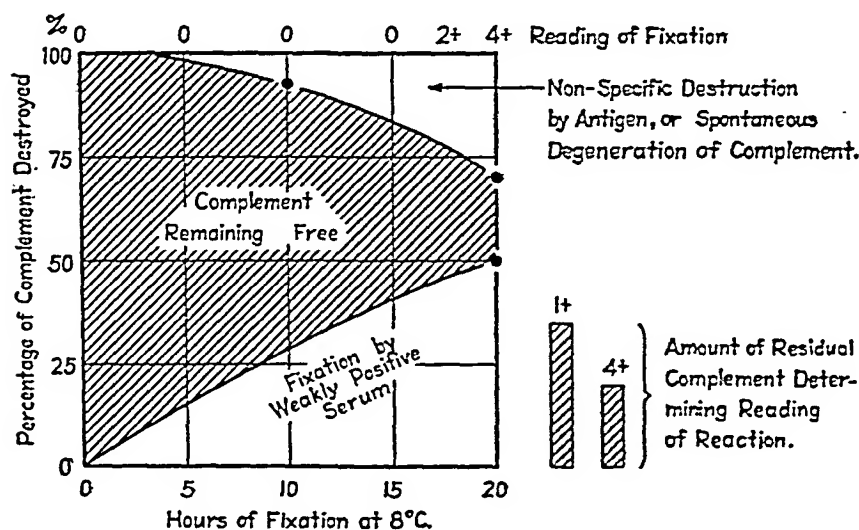


FIG. 2. Rôle of non-specific destruction of complement by antigen in increasing the sensitivity of Wassermann.

Inhibiting Effect of Serum upon Fixation

If a very strongly positive serum is titrated for its reagin content by water bath ($\frac{1}{2}$ hour at $37^{\circ}\text{C}.$) and ice box (3 hours) fixation, one obtains the results indicated in Table I. The sensitivity of the two methods is approximately the same, using these time intervals (Protocol 2, Table I).

Protocol 2

Two sets of tubes were set up as outlined in the heading to Table I. One was incubated at 37° for $\frac{1}{2}$ hour, the other at 8° for 3 hours, followed by $\frac{1}{2}$ hour at 37° .

more serum is used. The phenomenon, illustrated in Fig. 4, is often erroneously interpreted as due to native amboceptor. It has also been misnamed "complement deviation," with which it has nothing in common. The true Neisser-Wechsberg phenomenon of complement fixation is an unexplained inhibition of hemolysis, due to excess amboceptor: the zone under discussion is an inhibition of complement fixation due to excess serum, and is manifested by increased hemolysis.

Protocol 4

Two identical series of tubes were set up, each containing complement, antigen, and increasing quantities of a weakly positive serum. One series was incubated at 37°C. for $\frac{1}{2}$ hour, the other at 6°C. for 4 hours, followed by the 37°C. incubation. Degrees of complement fixation were then determined by adding sensitized cells. As is seen in Fig. 4, the inhibition by the larger quantities of serum at 37°C. is sufficient to make the serum appear completely negative, while in the ice box the inhibition is not nearly so pronounced.

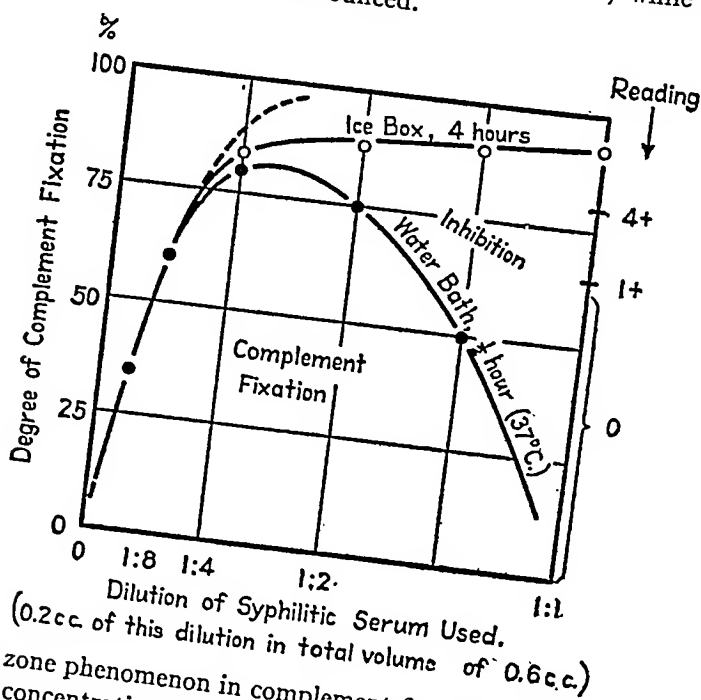


FIG. 4. The zone phenomenon in complement fixation: probably due to serum protein in high concentration in inhibiting fixation by a weakly positive serum.

the presence of normal serum has depressed the sensitivity to about 10 per cent of its original value. If this inhibiting effect of serum upon fixation is studied quantitatively at 8°C. and 37°C., one obtains the results summarized in Fig. 3 (Protocol 3).

Protocol 3

The reagin content of a very strongly positive serum, *i.e.*, the dilution in which it was still positive, was determined at both 8°C. (6 hours) and 37°C. ($\frac{1}{2}$ hour). The number of reagin units necessary to give fixation in the presence of added normal serum (0.2, 0.1, 0.05, and 0.025 cc.) was then determined at both temperatures. The results are summarized in Fig. 3.

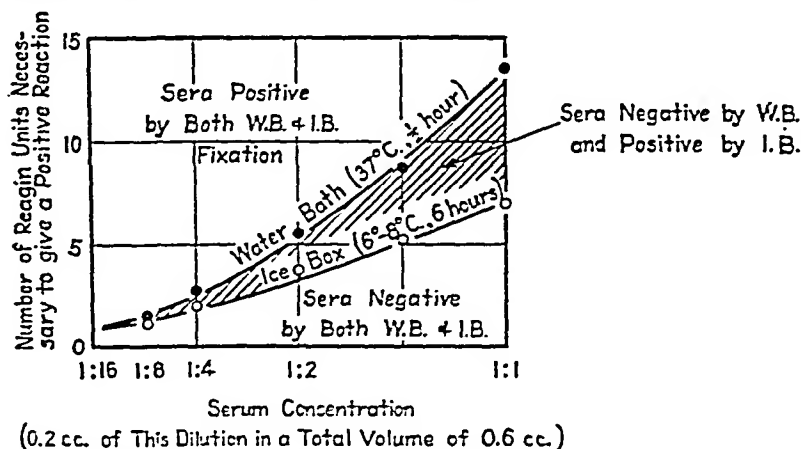


FIG. 3. Effect of normal serum upon sensitivity of the Wassermann reaction at 37° and 8°C.

The greater sensitivity of the ice box Wassermann is in part due to the fact that this inhibition due to the presence of serum is less marked at lower temperatures (Fig. 3). Even at the lower temperature, however, the inhibition is still sufficiently pronounced to make the test with 0.2 cc. of whole positive serum only slightly more sensitive than that with 0.1 or even 0.05 cc. of the same serum (*cf.* Fig. 4).

It is this extreme inhibition by concentrated serum which causes the so called "zone phenomenon" in complement fixation. A weakly positive serum may give a completely positive reaction when tested in *e.g.*, 1:5 dilution, but, paradoxically, appear completely negative when

Exactly the same result is obtained using any kind of antigen-antibody complex (sensitized bacteria or red cells; foreign protein-antiprotein precipitate).

The most probable explanation of this phenomenon is therefore that normal serum protein is adsorbed by the lipoid particles *after* they have combined with reagin, forming a protective film which prevents the adsorption (fixation) of complement.

SUMMARY AND DISCUSSION

Serum, in concentrations greater than 1:25, causes a marked inhibition of complement fixation in general and of the Wassermann reaction in particular. The serum protein is probably adsorbed by the colloiddally dispersed lipoid-reagin complexes, forming a protective film which prevents the fixation (adsorption) of complement. This inhibition explains the zone phenomenon in complement fixation: a weakly positive serum may give a completely positive reaction in *e.g.*, 1:5 dilution, and yet, because of this serum inhibition, may appear completely negative when tested as whole serum.

The greater sensitivity of the ice box test is due (1) to the fact that the serum inhibition just described is less marked at lower temperature; (2) to the prolonged incubation time, making for greater specific fixation; (3) to a more marked non-specific destruction of complement by antigen; and (4) a spontaneous deterioration in the longer ice box test.

Because of the inhibition by serum protein in high concentration, a quantitative Wassermann technique involving the use of graded quantities of serum is worthless when carried out at 37°C. Even the ice box test, which is less susceptible to this inhibiting effect, will yield a positive reaction with whole serum only when the circulating reagin exceeds a surprisingly high threshold (six to ten times the quantity which could be detected in dilute serum). It is well known that a negative Wassermann, even by a very sensitive test, does not exclude syphilis: it now appears that a negative Wassermann does not exclude circulating reagin.

Cause of Inhibition of Fixation by Serum (Zone Phenomenon)

The cause of this inhibition by serum is of theoretical importance only.

It has already been shown (Eagle, 1930, *b*) that complement fixation in the Wassermann reaction involves two distinct and consecutive reactions, (1) the combination of antigen with syphilitic reagin, which is deposited as a film of denatured protein around the colloiddally dispersed lipid particles of the antigen dilution, and (2) the adsorption of complement by this film of denatured reagin-globulin. As shown

TABLE III

Effect of Normal Serum upon Complement Fixation by Beef Heart Lipoid-Reagin Aggregates

Figures in body of table indicate percentage of complement fixation.

Syphilitic serum, cc.	0.4	0.2	0.1	0.05	0.025	0.0125	0.0062	0.0031	Apparent reagin titre (dilution in which serum gives 4+ = 80 per cent fixation)	Sensitivity
Negative serum added after combination of antigen with reagin										
cc.										
0				>90	90	65	35	20	28	100
0.025				>90	85	50	25		20	70
0.05			>90	75	40	25			16	55
0.1		>90	60	35					3	10
0.2	50	35							<1	<4

in the following experiment, the effect of serum is upon the second of these two reactions.

Antigen and syphilitic serum were allowed to react for 1 hour at 37°C. Varying quantities of negative serum and complement were then added and the degree of fixation determined after $\frac{1}{2}$ hour at 37°C. If excess serum acts to prevent the union of reagin and antigen, the added negative serum should have been ineffective; but if it prevents the adsorption of complement by the formed antigen-reagin complex, it should inhibit fixation as strongly as if all the reagents had been added simultaneously. As shown by Table III, the latter is the case.

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only, therefore, were the nitrogenous impurities of protein origin more difficult to remove, but also when this was accomplished the product was found to be contaminated by at least two substances which had not been encountered in the work on Type I, II, and III pneumococcus polysaccharides—a serologically inactive substance somewhat related to chitin, and the “C substance” first described by Tillett and Francis, and later studied by Tillett, Goebel, and Avery* (3). Since the chemical and physical properties of both of these products were very similar to those of the Type IV specific polysaccharide, removal of these and the other principal impurities was accomplished only after the sacrifice of much material in preliminary experimentation.

The none too satisfactory method finally adopted was as follows:

20 l. lots of autolyzed Type IV pneumococcus cultures in 0.3 per cent glucose-meat infusion-phosphate broth were autoclaved and concentrated on the water bath to 1 to 1.5 l. The resulting syrup was precipitated with alcohol up to 5 l. and allowed to stand overnight. The supernatant liquid was decanted and the precipitate centrifuged and taken up in the minimum amount of water. The solution was centrifuged and the supernatant and washings were treated with 20 per cent of alcohol and kept until the concentrate from a number of lots had accumulated.

The concentrate from about 80 l. of culture was reprecipitated with four volumes of alcohol and the precipitate taken up in water, diluted to 5 l., acidified with a few cubic centimeters of acetic acid, stirred mechanically, and precipitated with aqueous tannic acid solution containing 200 gm. of the acid per liter, adding the acid only as long as the precipitate was dark and heavy. 600 to 1000 cc. usually sufficed. After thorough stirring the mixture was allowed to stand overnight in the cold. The turbid supernatant was centrifuged in the cold until merely opalescent, while the main precipitate was ground in a mortar with water weakly acidified with acetic acid, filtered on a large Buchner funnel, and sucked dry. The filtrate and the main solution were concentrated *in vacuo* to 400 cc. and precipitated with 350 cc. of alcohol, bringing down a heavy syrup which contained little active material. The supernatant was drawn off and precipitated with alcohol up to 2 l. and allowed to stand. The supernatant was decanted and the precipitate centrifuged, yielding three layers, of which the middle one contained most of the specific substance. This was taken up in 250 cc. of water, and, after the addition of 50 gm. of sodium acetate, precipitated with five volumes of alcohol. The precipitate was dissolved in 1 l. of water, treated with 75 gm. of sodium acetate, acidified with

* The writers wish to express their gratitude to these workers for the private communication which led to the recognition of this substance in the crude product

SPECIFIC AND NON-SPECIFIC POLYSACCHARIDES OF TYPE IV PNEUMOCOCCUS*

BY MICHAEL HEIDELBERGER, PH.D., AND FORREST E. KENDALL, PH.D.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York)

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The recent separation of Group IV pneumococcus into a number of types with well defined specific properties (1) suggested a comparison of the specific polysaccharide of one of the new subdivisions with the highly type-specific and chemically distinct carbohydrate haptens of Type I, II, and III pneumococcus (2). It seemed of interest to determine whether the substance responsible for the specificity of one of the new types would resemble the specific polysaccharides of Type I, II, and III pneumococcus, or whether it would be as different from these as each of the three is from the others.

Type IV pneumococcus was accordingly chosen for study, since at the time it appeared to be one of the most important of the new subdivisions of Group IV (1). The cultures and homologous antiserum were kindly furnished by Dr. Georgia Cooper of the New York City Board of Health Laboratories, and the writers wish to express their heartiest thanks both to Dr. Cooper and to Dr. William H. Park for their interest and cooperation.

EXPERIMENTAL

It was evident from the very first that the isolation of the Type IV specific polysaccharide would be a very difficult matter since the hapten required far more alcohol for its precipitation than did the corresponding substances of Type I, II, and III pneumococcus. Not

* The work reported in this communication was made possible by the Harlax Research Fund of the Presbyterian Hospital.

All of the copper can be removed by repeated precipitation with acetic acid and alcohol, and the final product is isolated as a curdy, snow-white mass, as indicated above. The yields varied from 0.1 to 0.5 gm.

The A fraction contained both inactive polysaccharide and "C substance." It was dissolved in about 25 cc. of water, centrifuged if necessary, made up to 125 cc. with glacial acetic acid, and treated in the cold with alcohol in small portions until the heavy turbidity produced first showed signs of flocking. On centrifuging in the cold a precipitate (A_1) and a clear supernatant were obtained, the latter yielding "C substance" on precipitation with acetone (A_2). This was centrifuged off sharply, dissolved in 15 cc. of water, treated in the cold with 50 cc. of glacial acetic acid, then with redistilled alcohol to the first permanent turbidity. Traces of precipitate containing more A_1 were centrifuged off, and the precipitation completed with redistilled alcohol up to 250 cc. After letting stand in the cold the precipitate was centrifuged off, redissolved in a few cubic centimeters of water, and poured into 200 cc. of redistilled acetone, yielding a stringy precipitate which was collected and dried as in the case of the B fraction. The yields varied from 0.2 to 0.5 gm.

The A_1 fraction was dissolved in 25 cc. of water, centrifuged, and the solution treated with nitrous acid as in the case of the B fraction to remove as much "C substance" as possible, and precipitated with an equal volume of acetic acid and enough alcohol to yield a clear supernatant on centrifugation. The precipitate (A_{1a}) was now almost inactive serologically, while the supernatant, precipitated with more alcohol, yielded a small fraction containing all three substances. A_{1a} was redissolved in a few cubic centimeters of water and made up to 200 cc. with glacial acetic acid, giving a copious precipitate which was redissolved, reprecipitated with acetic acid and redistilled alcohol, dissolved once more in water, and isolated by pouring into redistilled acetone as in the case of the other fractions. Usually 1.0 to 2.5 gm. of this inactive polysaccharide was obtained.

Isolation of "C Substance" from Type I Pneumococcus Broth.—16 l. of Type I pneumococcus broth were worked up according to a recently published short method for the isolation of the specific polysaccharide (4). It was found that the supernatant from the first isoelectric precipitation gave strong precipitin reactions with Type I, II, and III pneumococcus antisera. The supernatant and the acetic acid washings of the SSS I were neutralized and concentrated to small bulk on the steam bath. The "C substance" was then precipitated by adding alcohol until the supernatant was free from reactive material. The precipitate was centrifuged off and dissolved in 0.01 N acetic acid. A small amount of Type I SSS remained undissolved. The solution was precipitated with alcohol and the precipitate tested against Type I, II, and III sera. Large amounts of SSS I seemed still to be present, and the precipitate was accordingly dissolved in water and the solution treated with saturated barium hydroxide solution until no further precipitate formed. The precipitate, consisting mainly of the salt of SSS I, was centrifuged off and the supernatant was freed from barium. The volume was now about 100

acetic acid, and precipitated with 50 gm. of thorium nitrate dissolved in water and made up to 100 cc. The mixture was centrifuged in the cold and the supernatant was run through a Sharples supercentrifuge for further clarification. The grayish, opalescent solution was then made alkaline with ammonia, and the copious precipitate, which came down readily on centrifugation, was washed with 1 l. of water. The water-clear supernatant and washings were acidified faintly with acetic acid and concentrated to small bulk *in vacuo*. The concentrate was dialyzed in a collodion bag,* with occasional concentration *in vacuo*, until practically free from nitrates, and was then again brought down to about 75 cc. *in vacuo*, treated with 75 cc. of glacial acetic acid, and then cautiously with alcohol in the cold until any precipitate formed just began to flock out. The precipitate (A) was collected by centrifugation and the supernatant further precipitated in the cold with alcohol, with the addition of acetone if necessary. The precipitate (B) was taken up in 20 to 30 cc. of water, centrifuged if necessary, and again treated in the cold with an equal volume of glacial acetic acid and small amounts of alcohol until the precipitate formed settled on centrifugation and left a clear supernatant. The precipitate was added to (A) and the supernatant precipitated with excess alcohol (B).

Since (B) represents crude Type IV polysaccharide its further purification will be taken up first. The precipitate is dissolved in about 30 cc. of water, concentrated partially *in vacuo* to remove alcohol, made up to the original volume, and treated with 3 cc. of 30 per cent aqueous sodium nitrite and 1 cc. of glacial acetic acid, with frequent shaking during the course of 1 to 2 hours. In dilute solution the "C substance" is destroyed by nitrous acid as was also found by Tillett, Goebel, and Avery, but much survives this treatment in the presence of relatively much Type IV polysaccharide. For further purification an equal volume of glacial acetic acid is added, and after removal of a very small initial precipitate with alcohol as before, alcohol is added up to 250 cc. The precipitate is centrifuged off sharply, taken up in a little water and reprecipitated twice with glacial acetic acid and redistilled alcohol, after which it is taken up in 20 to 30 cc. of water, poured into twenty volumes of redistilled acetone containing a little glacial acetic acid, centrifuged if necessary, collected on a hardened filter, and dried *in vacuo* over calcium chloride, paraffin, and crushed sodium hydroxide. If colored impurities persisted down to this point the appearance of the product could often be improved by solution in a little water, making up to 200 cc. with 6 per cent cupric acetate solution, precipitating in the cold with 200 cc. of chilled 20 per cent sodium hydroxide solution, centrifuging, taking up, with cooling, in a little water and acetic acid until permanently acid, centrifuging from the precipitate of copper acetate and washing this with a little ice-cold water, adding an equal volume of acetic acid to the supernatant and washings, and making up to 1 l. with alcohol.

* Considerable amounts of "C substance" pass through the bag at this stage, but not rapidly enough to afford a convenient method for its quantitative removal from the Type IV substance.

POLYSACCHARIDES OF TYPE IV PNEUMOCOCCUS

The sample, dissolved in 100 cc. of 12 per cent HCl, was heated to boiling over a small flame and distilled in a slow current of steam for 6 hours, about 500 cc. of distillate being collected. The concentration of HCl in the distillate was adjusted to 3 per cent, 2 cc. of 20 per cent potassium bromide solution was added and the

TABLE I
Summary of Properties of Type IV, "C," and Non-Specific Polysaccharide Fractions

Preparation	$[\alpha]_D$	Acid equivalent	Total N	NH ₂ N	Acetyl N	Reducing sugars on hydrolysis (as glucose)	Pentose	Phosphorus	Ash (as Ca)	C	H
			per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Type IV											
68 B	+33.9°	3200	5.3								
71 B	+34.1°	3330	5.4	0.4							
72 B	+31.3°	1403	4.7						0.1		
75 B	+29.2°	2310	5.9			76.4	14.6	<0.1	0.3		
76 B	+37.7°	1250	5.2	5.9		75.5	12.0				
78 B	+17.0°	1380	5.9	0.1	5.6		13.3		0.1		
79 B	+35.0°	1420	5.6			67.0	5.4		0.1		
						68.0	7.9	<0.1	0.0	45.9	6.7
"C" substance									0.2		
76 A ₂	+49.3°	1250	6.2	0.7	3.7						
78 A ₂	+33.0°	1000	6.6	1.1	3.7	37.0	5.0		0.9		
80 (from Pneumococcus I)	+35.9°	690	5.8			40.0	3.1		1.1	42.1	6.7
81 (from Pneumococcus III)	+51.2°	1270	5.8	0.8	3.7			4.0	<1.0		
					30.4						
Inactive fraction							4.0	<1.0			
75 A _{1a}	0°	5600	5.7								
76 A _{1a}	+20.2°	3970	6.0	0.0	58.5				0.0		
				5.6	52				0.1	46.9	6.7

titration made with 0.01 N potassium bromate solution, using starch-iodide solution as an outside indicator. A definite blue color 2 minutes after the addition of the last portion of bromate was taken as the end point. A blank was run upon an equal volume of 3 per cent hydrochloric acid containing the same concentration of potassium bromide, titrating it to the blue color taken as the end point in the

cc. The "C substance" was precipitated with alcohol, the precipitate dissolved in about 5 cc. of water and reprecipitated with five volumes of glacial acetic acid. The precipitate was centrifuged off and the supernatant precipitated with alcohol. The acetic acid precipitate consisted largely of SSS I and inactive material, while that precipitated from the acetic acid supernatant with alcohol gave strong reactions with Type III antiserum and a slight test with Type I antiserum. The acetic acid precipitation of the latter fraction was repeated four times, or until the more soluble fraction, dissolved in 5 cc. of water, no longer contained material insoluble in five volumes of glacial acetic acid. After repeated precipitation with redistilled alcohol and redistilled acetone it was filtered off and dried. The yield was 60 mg.

Isolation of the "C Substance" from Type III Pneumococcus Broth.—It was found that the alcoholic supernatants obtained in the isolation of the specific carbohydrate of Type III pneumococcus (5) gave strong precipitin reactions with Type I pneumococcus antisera and with Type III antisera that had been completely absorbed with Type III specific carbohydrate. The separation of the "C substance" from the dark nitrogenous impurities in the supernatants from the first two precipitations proved to be difficult. However, the supernatant from the first alkaline alcoholic precipitation (the third precipitation in the series) contained a considerable amount of the "C substance" and was accordingly used as a source of material. Upon acidifying with acetic acid a precipitate formed that gave a positive precipitin reaction while the supernatant was nearly negative. The precipitate was centrifuged off and then taken up in water. A small amount of insoluble material was centrifuged off and the supernatant precipitated with alcohol. This was repeated as long as a residue insoluble in water was obtained. Four alcoholic precipitations were required. The final precipitate was taken up in 1.5 cc. of water and 5 cc. of 10 per cent trichloroacetic acid were added. A precipitate formed and was centrifuged off. The supernatant was precipitated with alcohol. The precipitate was taken up in 5 cc. of water and precipitated with five volumes of glacial acetic acid. The "C substance" in the supernatant was precipitated with alcohol and the fractional precipitation with glacial acetic acid repeated twice as above. The substance was then freed from acetic acid by repeated precipitations with redistilled alcohol and was then precipitated by pouring into redistilled acetone. The yield was 100 mg. from 24 l. of broth culture.

The properties of the fractions are summarized in Tables I and II, while Table III gives comparative data for the specific polysaccharides of Type I, II, III, and IV pneumococcus and for the inactive and "C" substances.

Pentose was determined in the different preparations by a modification of the Pervier and Gortner method (6) further adapted as a semi-micro-method by the use of smaller apparatus and tubes of narrower bore.

pentose determinations. The amount of pentose was calculated as 0.01 cc. of *N* bromate $\times 0.7505$ = milligrams of pentose.

The method was checked by determinations of pentose in gum arabic and upon pure recrystallized xylose.

10.0 mg. xylose distilled 6 hours required 14.5, 14.6 cc. 0.01 *N* KBrO_3 . Blank 1.0 cc.

$13.55 \times 0.7505 = 10.17$ mg. pentose.

28.3 mg. gum arabic required 15.2 cc. 0.01 *N* KBrO_3 . Blank 1.0 cc.

$14.2 \times 0.7505 = 10.65$ mg. pentose = 37.6 per cent pentose.

14.3 mg. gum arabic required 8.0 cc. KBrO_3 . Blank 1.0 cc.

$7 \times 0.7505 = 5.25$ mg. pentose = 36.7 per cent pentose.

On some samples pentose and -uronic anhydride were determined at the same time by the method of Bowman and McKinnis (7) using the modified method of Pervier and Gortner for determining the pentose.

The sample, dissolved in 12 per cent hydrochloric acid, was distilled in a current of steam and carbon dioxide-free air. The furfural and steam were condensed in an ice-cooled receiver and the CO_2 was absorbed in 0.02 *N* barium hydroxide solution after passing through an absorption bulb containing silver nitrate to remove traces of hydrochloric acid.

The apparatus and method were checked upon a sample of pure, crystalline aldobionic acid from gum arabic (8).

100 mg. of $\text{C}_{12}\text{H}_{20}\text{O}_{11} \cdot 2\text{H}_2\text{O}$ required 28.15 cc. 0.02 *N* $\text{Ba}(\text{OH})_2$. Blank, 2.30 cc. $28.15 - 2.3 = 25.85 \times 0.44 = 11.32$ mg. CO_2 or 45.28 per cent -uronic anhydride. Calculated for $\text{C}_{12}\text{H}_{20}\text{O}_{11} \cdot 2\text{H}_2\text{O} = 44.9$ per cent.

Pentose: 650 cc. distillate; 300 cc. aliquots titrated. Required, 10.2, 9.7 cc. 0.01 *N* KBrO_3 . Blank 0.50 cc. $9.7 \times 0.75 = 7.27$. $9.2 \times 0.75 = 6.90$. Mean. 7.08 mg. pentose. $7.08 \times \frac{650}{300} = 15.34$ mg. pentose, or 46 per cent -uronic anhydride.

No CO_2 was evolved in the case of 78 B and 79 B.

The acetyl groups were determined by either of two methods: (1) by distilling from a 10 per cent sulfuric acid solution in a current of steam, maintaining the volume constant and titrating the distillate; (2) by distilling from a phosphoric acid solution containing 3 cc. of 85 per cent phosphoric acid. The flask was heated in an oil bath at 140° and the solution evaporated nearly to dryness between additions of 25 cc. portions of water. This method had the advantage of giving smaller blanks and smaller volumes of distillate for titration.*

* Private communication from Prof. Hans T. Clarke.

TABLE II

Specificity Tests with *Antipneumococcus Sera, Types II and IV*

0.5 cc. serum 2:3, 0.5 cc. dilution.

Preparation	Serum II	Serum IV	Preparation	Serum II	Serum IV	Preparation	Serum II	Serum IV	Preparation	Serum II	Serum IV
75 Λ_2 1:200	++±	±	76 Λ_{12} 1:1000	++±	++±	78 Λ_{12} Not tested					
1000	±	-	10,000	+	±	Λ_2 1:10,000	++	++			++
10,000	-	-	100,000	-	-	100,000	++±	++±			++
Λ_2 1:1000	++	++	Λ_1 1:1000	-	-	500,000	++	++			++
10,000	++±	±	100,000	++	++	1,000,000	++±	++±			++
100,000	+	-	500,000	++±	-	1,500,000	+	+			-
500,000	-	-	1,000,000	++±	++	2,000,000*	+	+			-
B 1:10,000	++±	++±	1,500,000	+	+	B 1:1000	±	±			++
100,000	±	+	2,000,000	+	±	10,000	-	-			++
1,000,000			B 1:1000	++	++	100,000	+	+			++
1,500,000			100,000	+	+	1,000,000	-	-			++
Λ_2 with nitrous acid, neutralized, diluted to 1:10,000			1,000,000	-	-	1,500,000	++	++			++
			2,000,000*			2,000,000*	±	±			++
B treated similarly, 1:10,000	±	++				79 B 1:1000	+	+			++
						10,000	++±	++±			++
						100,000	±	±			++
						500,000	+	+			++
						1,000,000	-	-			++
						1,500,000	±	±			++
						2,000,000*	±	±			++

Readings in parentheses obtained after centrifuging at low speed.
 All serum and dilution controls negative even after centrifuging.
 * Highest dilution tested.

hour's boiling $[\alpha]_D$ was $+7^\circ$ and the reducing sugar content 64 per cent. The solution was decolorized with norit and concentrated repeatedly *in vacuo*, first with the addition of methyl alcohol and finally with methyl alcohol and benzene in order to drive off the last traces of water. The resulting spongy mass was extracted twice with hot absolute ethyl alcohol, the total volume being 250 cc., and was almost entirely soluble. Neither fraction yielded glucosamine hydrochloride. On concentration of the solution to small bulk *in vacuo* it deposited 0.25 gm. of a solid. $[\alpha]_D^{25}$ was $+27.8^\circ$ (0.1134 gm. substance dried *in vacuo* at 61° , α , $+0.63^\circ$, $l = 1$, 5.0 cc.) and no mutarotation was observed.

Micro-Kjeldahl on 0.5 cc. of this solution: 2.62 cc. N/70 HCl used; N, 4.6 per cent.

Micro-Volhard on 0.25 cc.: 0.85 cc. N/50 AgNO₃ used. Cl, 10.6 per cent.

Reducing sugars: 0.5 cc. made up to 20.0 cc., 5 cc. samples, 0.8, 0.8 mg. glucose. As glucose, 28.2 per cent. After hydrolysis in 1.5 N HCl for 2 hours, 1.63 mg. glucose. As glucose, 57.5 per cent.

Since it seemed possible that more or less methyl glucoside had been formed after the addition of methyl alcohol to the original concentrate which contained an excess of hydrochloric acid, a portion was boiled for 2 hours with 1.5 normal hydrochloric acid, and it was indeed found that the reducing sugar content, calculated as glucose, rose to 57.5 per cent. This would be the equivalent of one reducing group in a substance of molecular weight 313, while the minimum molecular weight on the basis of the nitrogen percentage is 305, and on the basis of the chlorine 335. The material might thus consist chiefly of the hydrochloride of an aminodihexose or an aminohydrochloride derivative of a disaccharide composed of one molecule of pentose and one of hexose.

DISCUSSION

Owing to the relatively small amounts of the polysaccharides in the Type IV cultures and their relatively great solubility in alcohol—at least before their isolation—these substances were far more difficult to separate from accompanying protein degradation products than the specific polysaccharides of Type I, II, and III pneumococcus. Once this separation had been fairly well effected, there remained the problem of separating each of three nearly similar polysaccharides from the

The acetic acid in the distillate was identified by converting it into the silver salt with silver carbonate, isolating and identifying the crystalline silver acetate.

Silver acetate from SSS 73:* 0.0294 mg. gave 0.0250 gm. AgCl. Found: 64.0 per cent Ag. Calculated: 64.7 per cent Ag.

*Hydrolysis of the Inactive Polysaccharide (76A_{1a}).—*1.0 gm. of substance was dissolved in 50 cc. of normal hydrochloric acid, boiled under a reflux for 3 hours, treated with acid-washed norit, and filtered. $[\alpha]_{25}^D$ was $+43.8^\circ$ ($\alpha_D + 1.75^\circ$, $l = 2$). The solution was concentrated to small bulk *in vacuo*, chilled, and seeded with glucosamine hydrochloride, 0.15 gm. of the salt separating. For analysis it was recrystallized from water.

0.0743 gm., volume 5.0 cc., $l = 1$; initial α , $+1.37$, $[\alpha]_D^{23} +92.2^\circ$; final α , $+1.04^\circ$, $[\alpha]_D +70.0^\circ$. For glucosamine hydrochloride: Initial $[\alpha]_D^{23} +100^\circ$; final, $+72.5^\circ$ (9).

The filtrate from what is thus almost certainly glucosamine hydrochloride appeared to contain a mixture of nitrogenous sugar derivatives, but yielded no definite products on fractionation. The alcohol insoluble portion gave analytical figures in agreement with the dihydrochloride of a diaminotrisaccharide, an intermediate product analogous to the acid-resisting triglucosamine hydrochloride encountered by Karrer and Hofmann in the hydrolysis of chitosan by snail juice (10).

Like chitin and chitosan, the inactive polysaccharide is partially hydrolyzed by snail juice. It yields an insoluble product and a small proportion of soluble reducing sugars. The snail juice, however, did not destroy the specificity of the Type IV and "C" polysaccharides in spite of their chemical relationship to chitin.

Hydrolysis of the Type IV Specific Polysaccharide.—1 gm. of a mixture of several active fractions was dissolved in 50 cc. of normal hydrochloric acid and boiled under a reflux for 2 hours. The specific rotation, which was originally $+33.5^\circ$, dropped to 12° , and the reducing sugars,† calculated as glucose, rose to 65 per cent. After another

* This was a preparation of Type IV specific substance precipitated by alcohol from alkaline solution, and washed with alcohol in order to ensure absence of adsorbed acetic acid.

† By the Schaeffer-Hartmann micro-method, *J. Biol. Chem.*, 1921, 45, 543.

workers prepared their material from "R"-pneumococcus cells, and the discrepancies in the two sets of data are perhaps due to the presence of impurities derived from the cells on the one hand, and from the broth on the other. The definite, though small, fraction of the nitrogen now found to be reactive as amino nitrogen, is, however, better in keeping

TABLE III

Comparison of the Type-Specific, Species-Specific, and Non-Specifically Reactive Polysaccharides of Pneumococcus

Polysaccharide	$[\alpha]_D$	Acid equivalent	Total N	Amino N	Acetyl N	Hydrolysis products	
			per cent	per cent	per cent	calculated as glucose, per cent	
Type I	+300°	310	5.0	2.5	0	28	(Galacturonic acid) (Amino sugar derivative)
Type II	+74°	1250	0.0			70	Glucose
Type III	-33°	340	0.0			75	Aldobionic acid, glucose
Type IV*	+30°	1550	5.5	0.1	5.8	71	(Amino sugar derivative)
Species-specific ("C" substance)†	+42°	1050	6.1	0.9	3.7	36	Acetic acid (Amino sugar derivative)
Inactive†	+10°	4540	5.9	0.0	5.6	55	Phosphoric acid Acetic acid (Glucosamine) (Amino sugar derivative) Acetic acid

* Average values, omitting 68 B and 71 B. Calculated for $C_6H_9O_4NHCOCH_3$: N, 6.9 per cent; for $C_6H_{10}O_5 \cdot (C_6H_9O_4NHCOCH_3)_2$: N, 4.9 per cent.

† Average values.

with the fact first reported by Tillett, Goebel, and Avery that the substance is destroyed by nitrous acid. According to the analytical data, one nitrogen atom in every seven is subject to attack by nitrous acid, while four in every seven are protected by acetyl groups.

If the phosphorus in the "C" substance is present in its usual form of combination with sugars, that is, as phosphoric acid attached to two

mixture. That this was not rigorously accomplished is evident from the varying data on successive preparations presented in Table I and the cross reactions with immune serum noted in Table II. It will be seen that each preparation of a polysaccharide of Type IV pneumococcus was contaminated with more or less of the accompanying carbohydrates, but in spite of this, marked differences in the chemical and specific properties were discernible. The serologically inactive fraction has the lowest optical rotation and the highest carbon content of the three, is the weakest acid, is the least soluble in alcohol or acetic acid, and differs from the Type IV and "C" substances in yielding, on hydrolysis, crystals with the optical rotation of glucosamine. The Type IV specific substance, on the other hand, differs from the others in being the poorest in nitrogen and the richest in reducing sugars on hydrolysis, and in occupying an intermediate position as regards optical rotation, carbon content, and acidity. The species-specific, or "C" polysaccharide, is the highest in optical rotation and in total and amino nitrogen, and the poorest in reducing sugars yielded on hydrolysis, in its carbon content, and in its proportion of acetylated nitrogen.* It differs from the fully acetylated Type IV substance in being broken down by nitrous acid, and differs from all the hitherto investigated specific polysaccharides of *Pneumococcus* in containing phosphorus.

At the suggestion of Dr. Thomas Francis, Jr., of the Hospital of The Rockefeller Institute for Medical Research the "C" substance was analyzed for phosphorus, and 4 per cent of this element was actually found. The "C" substance is thus the first phosphorus-containing specific polysaccharide to be encountered. The phosphorus is firmly bound in organic combination, as no test for phosphate can be obtained with the molybdate reagent until the solution of the polysaccharide has been heated with acid. At 100° phosphoric acid is only slowly split off by normal hydrochloric acid or sodium hydroxide.

From Table I it is seen that the "C" substance, whether derived from Type I, III, or IV pneumococcus broth, showed a higher optical rotation, higher nitrogen, and in two cases a higher reducing sugar content on hydrolysis than reported by Tillett, Goebel, and Avery. These

* The acetyl groups are considered to be attached to nitrogen rather than to oxygen, since they survived treatment with strong alkali during the purification process.

chitin. Whether this fraction is derived from the broth or is a degradation product of the bacterial "skeletal" substance is not known; but this uncertainty does not apply to the Type IV specific substance, which is undoubtedly derived from the bacteria themselves. Thus the finding of one, or possibly two substances similar in structure to chitin, but not identical with it, may point toward the cause of the still existing uncertainty as to whether or not chitin is a constituent of the bacterial cell wall (12). Finally, owing to the presence of the "C" substance or species-specific polysaccharide in the crude material, it has been possible to extend the observations of Tillett, Goebel, and Avery (*loc. cit.*) and to contrast the properties of this substance with those of the type-specific polysaccharides of *Pneumococcus*.

SUMMARY

1. Three nitrogen-containing polysaccharides have been isolated from autolyzed cultures of Type IV pneumococcus: (1) a type-specific carbohydrate differing markedly from those of Type I, II, and III pneumococcus, and representing a type of substance hitherto not observed among specific polysaccharides, (2) a chemically similar carbohydrate without specific function, and (3) the "C" substance, or species-specific polysaccharide of Tillett, Goebel, and Avery.

2. The chemical differences between the specific polysaccharides of *Pneumococcus* are discussed, and the relationship of the new examples to chitin is pointed out and its bearing indicated on the unsettled controversy as to whether or not chitin occurs in bacteria.

3. The data of Tillett, Goebel, and Avery on the "C" substance have been extended.

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hydroxyl groups, with one acid group free, and if this represents the total acidity of the substance, 4 per cent of phosphorus would correspond to an acid equivalent of 775. The higher values found in three cases are possibly to be explained by the presence of basic ash.

The differences between the three polysaccharides isolated from Type IV pneumococcus are summarized in Table III, which also shows the corresponding data for the Type I, II, and III specific substances. The differences between the latter have already been discussed (11), but now that more material is available it is seen that the specific polysaccharides of *Pneumococcus* fall into two sharply defined groups: on the one hand, the Type II and Type III substances, which are nitrogen-free, and on the other, the Type I, Type IV, and "C" substances, which contain nitrogenous sugars. The last two examples in this group, with their content of acetylated nitrogen, are more closely related to chitin than is the Type I substance. In the nitrogenous group, the Type I substance differs sharply from the others in its high optical rotation, its pronounced amphoteric character, its insolubility at the isoelectric point, its freedom from acetyl groups,* and in its high proportion of nitrogen susceptible to attack by nitrous acid. The "C" substance differs from the other members of both groups in its phosphorus content, but resembles the Type I substance in that its specificity is destroyed by nitrous acid, and is somewhat similar to the Type IV substance in that a part of the nitrogen is acetylated. The Type IV specific substance, on the other hand, differs from the Type I and "C" substances in containing only acetylated nitrogen and in yielding as high a percentage of reducing sugars on hydrolysis as do the nitrogen-free Type II and Type III substances.

It is plain that the study of the Type IV specific polysaccharide has brought to light a carbohydrate of a type new amongst those with specific properties, but somewhat resembling chitin in its general structure. It has, moreover, again shown that in the closely related pneumococcus types thus far studied, each polysaccharide responsible for type specificity is radically different from the others in structure, composition, and properties. The study has also shown the presence in the culture of a serologically inactive polysaccharide, chemically similar to the Type IV substance, but even more closely related to

* Unpublished experiments.

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following reasons: first, after being washed free of soluble nickel, this suspension did not oxidize rapidly to the soluble form in the presence of air, moisture, and living tissue; second, it could be determined quantitatively by Fairhall's (4) potassium di-thio-oxalate method; and, finally, the difference between nickel determinations made before and after treatment with hydrofluoric acid represented not only a very accurate control of the normal nickel content of the sample being analyzed, but also furnished unquestionable evidence that absorption of particulate matter had occurred.

A quantity of garnierite dust, produced by grinding the mineral in a ball mill, was suspended in 2 l. of distilled water and allowed to settle for 15 to 20 minutes in an ordinary laboratory 2 l. cylinder. At the end of this time, the fluid was siphoned off down to the 20 cc. mark, thus eliminating the heavier and larger particles. The suspension collected was first concentrated and then washed by repeated centrifugalization in large centrifuge bottles until the wash water did not give the slightest test for nickel with potassium di-thio-oxalate. The sediment of particles was then resuspended in enough 0.85 per cent salt solution so that 1 cc. contained 12 mg. of nickel. After repeated washing very little adjustment was necessary to bring the hydrogen ion concentration between pH 7.2 and 7.6.

The dogs used were anesthetized by sodium barbital, 0.35 gm. per kilogram intravenously. They were killed by bleeding to death at the close of the experiment. The omentum was drawn out of the abdomen through a linear incision and floated freely in a dish of garnierite suspension kept at body temperature and stirred by an occasional gentle blast of compressed air. The thoracic duct was cannulated in all experiments and in three of the group making Table II, the right lymphatic duct was tied so that all lymphatic entrance into the circulation was blocked. The livers and mediastinal lymph nodes were removed, washed in distilled water, weighed, and analyzed. In every instance the ligamentous attachments of the liver were carefully removed, so that liver tissue alone was analyzed.

Control fresh tissue, except lymph, was secured from dogs recently killed by the officials of the city.

DISCUSSION

In two cases out of five the normal liver contained traces of nickel, too weak to measure accurately. All other normal tissues were negative.

In the six experimental animals in which the omentum was isolated from the peritoneal cavity and exposed to garnierite particles in suspension, the lymph from the thoracic duct was negative in five cases.

ABSORPTION OF PARTICULATE MATTER BY THE GREAT OMENTUM

By ALBERT P. BATCHELDER, MADELEINE E. FIELD, AND
CECIL K. DRINKER, M.D.

(From the Department of Physiology, Harvard School of Public Health, Boston,
Massachusetts)

(Received for publication, February 20, 1931)

Past attempts to determine the channels of absorption of particulate matter in the great omentum have depended on the use of suspensions made up of chemically inert, colored particles. The interpretation of results and the conclusions were based on the subsequent microscopic recognition of these particles *in situ*.

Shipley and Cunningham (1) floated the omentum of the cat (decerebrate) in a dish of filtered India ink and found particles of carbon free in the portal vessels soon afterward. They believed the omentum of the adult animal contained few if any lymphatics and that the particles in question were absorbed by the blood capillaries. Poynter (2) confirmed this finding. Higgins and Bain (3) in careful experiments upon cats were unable to find that omental capillaries were in any way concerned in the removal of particles. The problem as to whether non-motile substances of bacterial dimensions must be removed *via* lymphatics or whether they are at least to some degree removed by entrance into blood vessels is of considerable practical importance and bears directly upon the problem of the real function of the lymphatics. We have consequently made observations very similar to those of Shipley and Cunningham but have used a particulate suspension chemically quantifiable so as to render the fact of absorption absolutely final. Even the most careful histological search can cover but a small part of the liver. If, however, the end-point is chemical the whole organ may be examined.

EXPERIMENTAL

A suspension of garnierite, an amorphous silicate of nickel and magnesium, $H_2(NiMg)SiO_4$, was used as particulate matter for the

The lymph from Animal 6 contained a trace of nickel estimated as 0.01 mg. in 1000 gm. lymph.

The liver findings were relatively uniform, ranging between 0.07 and 0.097 mg. of nickel per 1000 gm. of fresh tissue. The blood from four animals and the mediastinal tissue including the lymph nodes from three were all negative. In four of the six liver determinations in which nickel was found in a concentration between 0.07 and 0.09 mg. per 1000 gm. of tissue, no trace of nickel could be found before the material had been treated with hydrofluoric acid. Therefore, the total amount of nickel must have existed as insoluble nickel silicate and must have been absorbed by the omentum as particulate matter. In one other case a trace of nickel could be detected before treatment with hydrofluoric acid. This trace might have represented oxidation of the garnierite during the 5 hour experimental period or, more likely, it represented the normal content of nickel of that particular liver sample.

The results indicate a certain amount of vascular absorption of nickel particles. Since Higgins and Bain failed to find particles histologically, we are inclined to believe the particles removed *via* the blood stream were very small, probably less than 1 micron in diameter. The suspension employed necessarily contained many particles of this degree of fineness. Key (5) considers that in absorption of particles from joints a small amount of material is taken by the blood vessels and that this consists of particles just at the limits of microscopic vision. When the omentum is examined at the close of such an experiment it is laden with particle-containing phagocytes. It would seem that if there had been any considerable migration of these cells into the omental capillaries, the analyses of the liver must have shown far higher figures for nickel than were obtained. The method by which inert particles deposited in the tissues enter closed lymphatic capillaries is unknown, but it is certain such entrance can occur without the aid of free-moving phagocytes. A similar sort of direct entrance into the blood stream is probably involved in the experiments we have described. It is clearly not an important way of getting rid of foreign particles, but it is certainly an existing one.

Field and Drinker (6), in a group of experiments dealing with the paths of absorption of horse serum, found that when the thoracic and

TABLE I
Nickel Content of Lymph, Blood, Mediastinal Lymph Nodes, and Liver in Normal Dogs

No. of animal	Nickel content per 1000 gm.			
	Lymph	Blood	Mediastinal lymph nodes	Liver
1	Neg.	Neg.	Neg.	Trace
2	—	Neg.	Neg.	Neg.
3	—	Neg.	Neg.	Neg.
4	—	Neg.	Neg.	Trace
5	—	Neg.	Neg.	Neg.

TABLE II
*Nickel Content of Lymph, Blood, Mediastinal Lymph Nodes, and Liver after Floating the Omentum in a Suspension of Nickel Silicate (Garnierite) for Varying Periods of Time**

No. of animal	Length of experiment	Nickel content in mg. per 1000 gm. tissue (before and after treatment with hydrofluoric acid)					
			Control lymph	Experimental lymph	Blood	Mediastinal lymph nodes	Liver
1	4 hrs.	Before	Neg.	Neg.	—	—	Neg.
		After	Neg.	Neg.	—	—	0.070
2	2½	Before	Neg.	Neg.	—	—	Neg.
		After	Neg.	Neg.	—	—	0.031
3	5	Before	Neg.	Neg.	Neg.	Neg.	Trace
		After	Neg.	Neg.	Neg.	Neg.	0.097
4	4	Before	Neg.	Neg.	Neg.	Neg.	Neg.
		After	Neg.	Neg.	Neg.	Neg.	0.077
5	5	Before	Neg.	Neg.	Neg.	Neg.	Neg.
		After	Neg.	Neg.	Neg.	Neg.	0.037
6	1	After	Neg.	(0.01)	Neg.	—	0.035

* Thoracic duct cannulated in all cases. Right lymphatic duct tied in Animals 2, 3, and 6.

right lymphatic ducts were tied horse serum injected subcutaneously did not reach the blood in periods as long as 7 hours, but that horse serum injected intraperitoneally could be detected in the blood in less than an hour. This result is similar to and confirmatory of that obtained with the nickel suspension.

SUMMARY

When the omentum of the dog is floated in a suspension of insoluble nickel silicate in physiological saline under circumstances precluding the possibility of lymphatic drainage, the liver removed after at least 1 hour contains nickel which must have been brought to it by way of the blood capillaries in particulate form. The blood capillaries are, therefore, a pathway for absorption of solid material but are not important in this respect.

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obtained concerning increased capillary permeability in inflammation and the consequent localization of bacteria when reaching a point of injury from the circulating blood stream. Previous investigators (8, 11) had studied the dissemination of bacteria from the site of inflammation by recovering the organisms in the blood stream. The local state of affairs was not studied. In the experiments reported in this paper evidence is presented to show that the failure of bacterial dissemination from the site of inflammation is due to fixation *in situ* of the microorganisms.

Fixation of Particulate Matter at the Site of Inflammation

An acute inflammatory reaction was induced in the foreleg of rabbits by the subcutaneous injection of 5 cc. of a mixture of 5 per cent aleuronat and 3 per cent

TABLE I

Retention of Carbon Particles (India Ink) at the Site of Subcutaneous Inflammation

Experiment	Duration of inflammation	Presence of carbon particles in axillary lymph node draining normal area	Presence of carbon particles in axillary lymph node draining inflamed area
	<i>hrs.:min.</i>		
1	6:45	+	0
2	22:00	Trace	0
3	23:30	+	0
4	29:00	+	0
5	50:00	++	0
6	74:00	+	0

starch in 0.5 per cent saline solution. The injection was made 2 or 3 cm. from the shoulder joint. After a variable interval of time, 3 cc. of diluted India ink was injected into the site of inflammation. The same amount was injected into the other leg to act as control. After a variable time the animal was etherized and both axillary lymph nodes excised and examined for the presence of carbon deposits.

The results of these experiments are shown in Table I. It is clear that whereas carbon particles injected into normal tissue passed to the tributary lymph nodes, this was not true when they were injected into an area of subcutaneous inflammation.

The attempt was next made as in experiments with other foreign

STUDIES ON INFLAMMATION

VII. FIXATION OF BACTERIA AND OF PARTICULATE MATTER AT THE SITE OF INFLAMMATION*

By VALY MENKIN, M.D.

(From the Department of Pathology, Harvard University Medical School, Boston)

(Received for publication, January 28, 1931)

In previous communications (1, 2, 3, 4, 5, 6) it has been shown that foreign substances, including a vital dye, a ferric salt, and a foreign protein, injected into an inflamed area were fixed *in situ* and failed to reach the regional lymphatic nodes as readily as under ordinary circumstances. When these same substances were injected into the circulating blood stream, they rapidly entered the site of inflammation. The accumulation of these substances in inflamed areas was doubtless associated with increased capillary permeability (2). The fixation of the foreign substances was shown to result primarily from mechanical obstruction, caused by thrombosed lymphatics and by a fine network of fibrin at the site of inflammation (7).

Pawlowsky (8) demonstrated that the dissemination into the blood stream of staphylococci injected into a previously inflamed knee joint was either inhibited or wholly prevented. The studies of Issayeff had shown that peritonitis caused by a sterile irritant increased temporarily the resistance of the animal to subsequent intraperitoneal inoculation of bacteria (9). Willis (10) studied the dissemination of tubercle bacilli from the site of cutaneous inoculation and showed that in reinjected guinea pigs the spread of tubercle bacilli from the site of inoculation is retarded, whereas in normal animals the organisms pass readily to the regional lymph nodes. Recently Opie (11) has shown that acute inflammation of the peritoneal cavity caused by aleuronat retards the rush of injected hemolytic streptococci from the peritoneal cavity into the circulating blood and after enduring 24 hours completely prevents it.

In this communication further observations are presented concerning the behavior of particulate matter and of bacteria at the site of inflammation when injected intravenously or directly into an inflamed area. It is believed that by such studies valuable information may be

*Read before the American Association of Pathologists and Bacteriologists, Cleveland, April 2, 1931.

disseminate rapidly to the restrosteral lymph nodes, fail to do so, at least to any marked degree. Histological section of the restrosteral lymphatic nodes of control animals show in the sinuses large deposits of carbon which are not phagocytosed. These particles evidently reach the restrosteral nodes by way of the lymphatic channels as free particles not contained in phagocytic cells.

When it had been demonstrated that carbon particles failed to disseminate to the tributary lymphatics from the site of inflammation, studies were undertaken to determine whether graphite particles injected into the circulating blood stream would rapidly enter an inflamed area.

TABLE III

Accumulation of Graphite Particles at the Site of Inflammation

Experiment	Duration of inflammation	Presence of graphite in inflamed areas	Presence of graphite in normal areas
	<i>hrs.:min.</i>		
1	5:30	+	0
2	6:15	Trace	0
3	6:20	+++	0
4	20:00	++	0
5	25:00	+	0
6	28:00	++	0
7	48:30	+++	0

Areas of acute inflammation were induced by injecting 0.1 to 0.2 cc. of 10 per cent croton oil in olive oil into the skin of the abdomen of a rabbit. Several hours later 10 cc. of graphite suspension diluted in 0.9 per cent saline was injected into the ear vein. After a variable interval of time the animal was etherized and the inflamed as well as the normal skin areas were studied for the presence of graphite deposits both by gross and by subsequent histological examination. In two experiments (Nos. 2 and 6) *Staphylococcus aureus* instead of croton oil was used as the inflammatory irritant. In Experiment 3 inflammation was induced by subcutaneous injection of concentrated broth into the extensor surface of the foreleg about 2 cm. from the shoulder joint.

The results of these experiments are shown in Table III. The inflamed cutaneous areas displayed in a striking manner graphite deposits usually at the periphery of a central necrotic region. Histological examination of such inflamed areas of about 24 hours duration

substances (1, 2, 3, 4, 5, 6) to determine whether India ink injected into the circulating blood would rapidly enter the inflamed area. No satisfactory evidence to that effect could be obtained. It is well known in this connection that India ink adheres to the endothelial lining of capillaries. Kusnetzowsky (12), however, recently reported that as the vessels became more permeable in areas of inflammation particles of India ink may make their way through the endothelial wall into the extracapillary spaces where they are taken up by the polyblasts.

Graphite ink (Hydrokollag "300") has the advantage over India ink of not adhering to the vessel wall (13). For this reason experi-

TABLE II
Retention of Carbon Particles in Inflamed Peritoneal Cavity

Experiment	Interval between injection of irritant and that of graphite	Total duration of inflammation	Presence of graphite in the retrosternal lymph nodes after its injection into inflamed peritoneal cavity	Presence of graphite in the retrosternal lymph nodes after its injection into normal peritoneal cavity
	<i>hrs., min.</i>	<i>hrs., min.</i>		
1	3:30	6:20	Trace to +	++
2	19:00	21:30	0	+++
3	20:30	22:50	0	++
4	22:00	24:00	+	+++
5*	22:00	23:30	Faint trace	++
6*	23:00	24:30	0	++ to +++

* Carbon particles injected in the form of diluted India ink.

ments were performed to determine whether such particles accumulate and are fixed in an inflamed area.

An acute inflammation of the peritoneal cavity was induced in rabbits by the injection of 10 cc. of an aleuronat suspension. After a variable interval of time 5 cc. of diluted graphite ink was injected into the inflamed peritoneal cavity of the experimental animal. The same quantity was injected into the peritoneal cavity of a normal rabbit to serve as control. Several hours later the animal was killed with ether and the retrosternal lymphatics and nodes examined for the presence of graphite deposits. Two experiments were also performed with India ink.

The results are shown in Table II. It is evident that with acute inflammation in the peritoneal cavity carbon particles, which normally

Fixation of Bacteria at the Site of Inflammation

Experiments were devised to determine whether bacteria would be fixed like carbon particles by the inflammatory reaction. The organism studied was *Bacillus prodigiosus*. In a few experiments *Bacillus pyocyaneus* was used. The selection of these microorganisms was due to the ease with which colonies on solid media could be identified for counting by the characteristic red or green pigment they produced.

An inflammatory reaction was induced in rabbits by the intraperitoneal injection of 10 cc. of an aleuronat suspension. After a variable interval of time 1.5 to 3 cc. of a saline suspension of *B. prodigiosus* was injected into the peritoneal cavity.

TABLE IV

Presence of B. prodigiosus in Retrosternal Lymph Nodes after Intraperitoneal Injection

Experiment	Interval between injection of irritant and that of bacteria	Total duration of inflammation	Number of colonies recovered from retrosternal lymph nodes	
			After injection of bacteria into inflamed peritoneal cavity	After injection of bacteria into normal peritoneal cavity
	<i>hrs.:min.</i>	<i>hrs.:min.</i>		
1	4:10	6:00	6	150
2	15:05	21:10	3	47
3	15:30	21:40	2	38
4	22:45	25:45	0	Innumerable
5	26:18	29:50	39	Innumerable
6	24:45	40:10	7	Innumerable

Several hours later the animal was killed with ether and the retrosternal lymphatic nodes removed under aseptic precaution. These were ground with 1 cc. of physiological saline. 0.1 cc. of this extract was inoculated in each of two agar slants by means of a sterile pipette. Uniform distribution of the inoculated material was secured by reclining the tubes horizontally for about 30 minutes before placing them in the incubator. After 24 hours the tubes were exposed to light at room temperature to accelerate the development of the characteristic pigment of the colonies. As control to this experiment an equal amount of the same saline suspension of *B. prodigiosus* was injected into the peritoneal cavity of a normal rabbit after which precisely the same technique was used as in the experimental animal in culturing the retrosternal lymphatic nodes. The colonies appearing on the surface of the tubes were in many cases easily counted. When they were very numerous, they were estimated by wrapping about the tube a piece of paper in which square

showed the graphite particles to be completely taken up by the cells of the inflammatory reaction. It was of some interest to note that these particles were in most instances phagocytosed by polymorphonuclear leucocytes. Within the lumen of blood vessels a number of leucocytes loaded with graphite particles were often observed. The question arose whether graphite particles injected into the circulating blood are brought to the site of inflammation entirely by leucocytes or whether capillary permeability is sufficiently increased to allow also some of these relatively large particles to pass through the endothelial wall. To settle this point histological examinations were made of skin areas in which the duration of inflammation was only about 6 hours (Experiments 2 and 3). Careful studies under oil immersion magnifica-

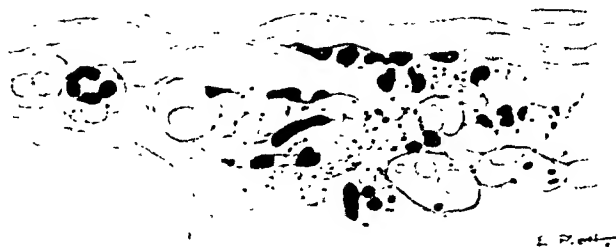


FIG. 1. Camera lucida drawing, showing passage of graphite particles through capillary walls into area of inflammation (about 6 hours duration). Magnified approximately 970 X.

tion revealed many capillaries with free graphite particles within their lumina. Some of these particles seemed evidently in the process of passing into the extracapillary spaces. In the tissue spaces many of these isolated graphite granules were found surrounding the capillaries. Only occasionally in such regions could carbon particles be seen within polymorphonuclear leucocytes. An area of this kind is illustrated in the accompanying drawing (Fig. 1). It is evident therefore that where the inflammatory reaction is of short duration the passage of non-phagocytosed graphite particles through the capillary wall can be readily demonstrated. Since no such evidence is obtained in sections of normal areas, the observations indicate that with inflammation the capillaries become permeable to particulate matter.

of definite size cut in a sheet of paper wrapped around the agar tube. Experiment 3 represents results obtained from a rabbit which had received an intraperitoneal injection of aleuronat followed by 3 cc. of *B. prodigiosus* suspension. An equal amount of bacterial suspension was injected into the normal peritoneal cavity of a control rabbit. At the end of the experiment a part of the mesentery of each animal was removed, weighed, ground, and inoculated on agar, as already described above. To correlate the results, the restrosteral lymphatic nodes were also removed in both of these rabbits. (See data of Experiment 1, Table IV).

As can be seen from the results recorded in Table V, the number of colonies recovered was consistently greater from the site of inflammation than from the corresponding normal area. It is to be noted that precisely similar results had been obtained with a foreign protein (4). These observations therefore represent direct evidence that bacteria are retained *in situ* by the inflammatory reaction, and consequently fail to disseminate to the tributary lymphatic nodes as readily as under ordinary circumstances.

The Accumulation of Bacteria at the Site of Inflammation

It has been known that injury to tissue may determine the localization in such areas of bacteria or ultrafilterable organisms which may be present in the circulating blood.

Calmette and Guérin (14) found that after the back of a rabbit had been shaved, intravenous injection of the virus of vaccinia resulted in a localization of the virus in the epilated area. Kettle (15) showed that tubercle bacilli injected intravenously in mice or rabbits tend to settle in localized subcutaneous lesions produced by various agents which cause increased vascularity and tissue necrosis. Chesney, Turner, and Halley (16) found that when rabbits with wounds in their backs are inoculated either intratesticularly or intravenously with *Treponema pallidum*, syphilitic lesions invariably develop in the wounds. Findlay (17) believed that histamine-like substances liberated at the point of injury are the specific agents which by their action on the capillary wall cause organisms present in the blood stream to localize in such an area. Sager and Nickel (18) recently demonstrated that subcutaneous injection of silver nitrate in rabbits causes sterile abscesses. After intravenous injection of green streptococci these organisms were recovered from the abscesses in some rabbits. They concluded that bacteria may become localized in places of lowered resistance. Quednau (19) pointed out that bacteria, especially pneumococci, in the blood stream tend to localize readily in areas of cerebral softening.

In previous studies (1 to 6) it has been shown by the writer that various foreign substances do not merely accumulate from the blood

openings of various sizes had been cut; the colonies in these square areas were counted through a magnifying glass.

The results are shown in Table IV. It is clear that in every experiment the number of colonies recovered from the retrosternal lymphatic nodes draining a normal peritoneal cavity was much larger than the number of colonies from the nodes draining an inflamed peritoneal cavity. The acute peritoneal inflammation evidently prevents the rush of injected bacilli to the tributary lymphatic nodes. These observations are in agreement with the results of Opie mentioned above on injected hemolytic streptococci (11).

TABLE V

Retention of B. prodigiosus at Site of Subcutaneous Inflammation

Experiment	Interval between injection of irritant and that of bacteria	Total duration of inflammation	Number of colonies recovered	
			Inflamed area	Normal area
	<i>hrs:min.</i>	<i>hrs:min.</i>		
1	1:30	3:30	175	37
2	4:00	6:00	225	175
3*	4:10	6:00	250	125
4	26:00	30:00	115	8
5	26:18	29:50	50	6

* The site of inflammation was located in the peritoneal cavity; see Experiment 1, Table IV, for related data on retrosternal lymph nodes.

Experiments were set up to determine whether this failure of bacterial dissemination to the tributary lymph nodes was actually due to retention of the microorganisms at the site of inflammation.

3 cc. of an aleuronat saline suspension was injected subcutaneously into the extensor surface of the foreleg of a rabbit, 2 or 3 cm. from the shoulder joint. After a varying interval of time 0.25 cc. of a saline suspension of *B. prodigiosus* was injected into the inflamed area. An equal quantity of this bacterial suspension was injected in the corresponding normal area of the opposite foreleg to serve as control. A few hours later both the inflamed and the normal areas were removed under aseptic precaution. Each area was weighed in a sterile petri dish, and then ground in a mortar with sand and a volume of saline corresponding to ten times its weight in grams. The extract was diluted ten times with sterile saline and 1.0 cc. of this dilution was inoculated in each of two agar slants. The number of colonies was obtained, as described above, by counting them through an aperture

The numbers of colonies recovered from inflamed and normal areas are shown in Table VI. It is clear that the number of intravenously injected bacteria accumulating at the site of inflammation is distinctly greater than in normal skin areas. These observations are in agreement with the results described above where it was demonstrated that capillary permeability in inflamed areas is sufficiently increased to allow graphite particles to pass through the endothelial wall.

The Failure of B. prodigiosus to Penetrate into an Inflamed Area When Injected at Its Periphery

In previous communications (5, 7) it was shown that trypan blue injected at the periphery of an inflamed area failed to enter it. In addition, microscopic studies revealed the presence of a network of fibrin within the tissues and numerous thrombosed lymphatics at the site of inflammation. Trypan blue injected into such an area is fixed *in situ* and fails to drain into the tributary lymphatics (1). The failure of the dye to enter the site of inflammation when injected at its periphery, its inability to escape from such an area when introduced directly into it, and the microscopic picture are all evidences that fixation of foreign substances is caused by mechanical obstruction at the site of inflammation in the form of thrombosed lymphatics associated with a fibrinous network.

To substantiate this point further two experiments were performed for the purpose of ascertaining whether *B. prodigiosus* injected at the periphery of an inflamed area would also (like trypan blue) fail to penetrate into it.

An area of inflammation was caused by the subcutaneous injection of 2 cc. of aleuronat suspension on the abdomen of each of two rabbits. 15 hours later this area displayed the usual signs of acute inflammation. 0.25 to 0.3 cc. of a saline suspension of *B. prodigiosus* was injected into five or six areas of the skin immediately surrounding the site of inflammation. In a normal skin area of the abdomen of the same size as the inflamed area similar injections were made to serve as controls. 6 hours later the animals were killed under aseptic precautions. The inflamed and normal areas were removed, weighed, and ground with sand and a volume of saline equal to ten times their weight. The extracts were diluted ten times and 0.1 cc. of the diluted saline extract inoculated in agar tubes as described above. There arose the possibility that the failure of *B. prodigiosus* to enter the site of inflammation might be due to the rapid circulation at its periphery, which

stream in an inflamed area, but also that these substances are held in such an area and are unable to drain readily through the regional lymphatics. The accumulation of these substances at the site of inflammation is doubtless in part the result of increased passage of fluid from the circulating blood, but observations on fixation show that at the same time their escape from the site of inflammation is mechanically retarded by the presence of a network of fibrin and by the occlusion of lymphatic vessels (7).

Experiments were undertaken to determine whether bacteria, that are fixed *in situ* when injected directly into an inflamed area, would ac-

TABLE VI

Accumulation of B. prodigiosus and B. pyocyaneus at the Site of Cutaneous Inflammation

Experiment	Duration of inflammation	Number of colonies recovered	
		Inflamed skin area	Normal skin area
	<i>hrs.-min.</i>		
1	2:50	5	1
2	4:00	5	2
3	4:00	43	4
4	4:52	25	6
5*	2 to 6 hrs.	24	5
6*	5:10	52	23
7**	8:50	75	13

* *B. pyocyaneus* injected.

** Croton oil and concentrated broth used as inflammatory irritant.

cumulate rapidly at the site of inflammation when injected into the blood stream.

Two or three areas of inflammation were induced by the injection of 0.5 cc. concentrated broth into the skin of the abdomen of rabbits. After a short interval of time 3 to 7 cc. of saline suspension of either *B. prodigiosus* or, in two experiments (Nos. 5 and 6), *B. pyocyaneus* was injected intravenously. After several hours the animal was killed with ether. The skin of the abdomen was treated first with 95 per cent, then with 70 per cent alcohol, and finally with sterile distilled water. Inflamed and normal skin areas were removed and weighed separately in sterile petri dishes. An extract of each area was made by grinding it in a mortar with a volume of saline equal to twice its weight in grams. Agar tubes were inoculated with these extracts as described in preceding experiments.

as 30 minutes after injection of the inflammatory irritant. This would indicate that the earliest change in inflammation is an increase in capillary permeability which permits the passage of fibrinogen from the plasma into the tissue spaces. The rapid formation of a fibrin network and of thrombi in lymphatics at the site of inflammation circumscribes the irritating substance and thus prevents its passage into the blood stream. This allows of a definite interval of time for the leucocytes to assemble for phagocytosis. The initial fixation of bacteria or of other injurious substances at the site of inflammation thus becomes a protective mechanism and plays a definite rôle in immunity. Rich (20) has questioned the view that acute inflammation *per se* can prevent the spread of bacteria. The observations presented in this communication show definitely that a non-specific inflammatory reaction retards the dissemination of bacteria for several hours at least (see Tables IV and V).

Recently Underhill and his collaborators (21) have shown that whereas trypan blue injected intravenously rapidly penetrates into the edematous part of a skin burn, the reabsorption of the dye from such an area is a very slow process. On the basis of their observations these investigators conclude that increased capillary permeability in one direction may exist simultaneously with decreased capillary permeability in the opposite direction. The experimental evidence presented in the first two papers (1 and 2) of this series although not disposing of the contention renders such an inference perhaps unnecessary. In our studies of lymphatics draining an inflamed area it was readily demonstrated that the failure of the dye to be reabsorbed from such an area was due to its fixation *in situ* by the mechanism discussed above.

The frequent localization of bacteria from the blood stream in a *locus minoris resistentiae* after preliminary trauma or other tissue injury is well known to pathologists and clinicians. The observations reported in this paper on the accumulation of bacteria in an inflamed area from the circulating blood stream may explain the mechanism of this phenomenon in terms of increased capillary permeability with resulting accumulation and fixation of bacteria at the point of injury.

would carry off the microorganisms faster than in a corresponding normal area. To rule out this objection, the skin at the periphery of the site of inflammation and the skin at the site of bacterial inoculations in the normal area were removed and cultured for the presence of the microorganisms.

The results of these two experiments were as follows:

Experiment	Number of colonies recovered			
	Inflamed area	Normal area	Periphery of inflamed area	Periphery of normal area
1	52	375	275	225
2	7	250	275	300

It is evident that whereas *B. prodigiosus* penetrated freely into the normal skin areas, considerably fewer organisms entered the site of inflammation. Furthermore, the number of colonies at the periphery of inflamed areas compared to that at the periphery of normal areas does not indicate that changes in velocity of circulation play a significant part in preventing *B. prodigiosus* from penetrating an inflamed area when injected at its periphery. These findings, in agreement with previous results obtained with trypan blue, furnish evidence that the fixation of bacteria in an area of inflammation is primarily the result of mechanical obstruction.

DISCUSSION

The experiments reported in this paper present further proof that various foreign substances, including a dye, a metallic salt, a foreign protein, particulate matter, and bacteria injected into the site of inflammation are fixed *in situ* and fail to drain readily into the tributary lymphatic vessels. These same substances injected intravenously accumulate rapidly in inflamed areas. This accumulation is partly associated with increased capillary permeability, but there is reason to suppose that it is also the result of the inability of these substances to escape from the site of inflammation owing to the presence of a fine network of fibrin and of thrombosed lymphatics. The mechanism of fixation takes place extremely early in the inflammatory process. In previous experiments (1) fixation of a dye at the site of inflammation could be demonstrated by studying the regional lymphatics as early

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CONCLUSIONS

India ink or graphite particles injected into an area of inflammation fail to disseminate to the tributary lymph nodes. When injected into a normal peritoneal cavity they rapidly appear in the retrosternal lymph nodes. When injected into an inflamed peritoneal cavity they are fixed *in situ* and fail to reach the regional lymph nodes.

Graphite particles injected in the circulating blood stream enter an inflamed area both as free particles owing to increased capillary permeability and also as phagocytosed material within leucocytes.

Bacteria (*B. prodigiosus*) injected into inflamed tissue are fixed at the site of inflammation and fail to disseminate to the regional lymph nodes as readily as when injected into normal tissue.

Bacteria (*B. prodigiosus*) injected at the periphery of an inflamed area do not readily penetrate into the site of inflammation. The experiments furnish evidence, in addition to that already provided, that fixation of foreign substances by the inflammatory reaction is primarily due to mechanical obstruction caused by a fibrin network and by thrombosed lymphatics at the site of inflammation.

Bacteria (*B. prodigiosus* and *B. pyocyaneus*) injected intravenously rapidly enter an inflamed area. It is suggested that localization of bacteria in a *locus minoris resistentiae* may be explained as the result of increased capillary permeability with subsequent accumulation and fixation of bacteria from the blood stream at the point of injury.

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Strains from different sources were used, without variations in results.

The cultures were centrifuged and washed with either Ringer solution, or physiological saline solution, and were then resuspended in just sufficient Ringer solution to give a fairly thick emulsion of bacteria. Samples were taken to determine the dry weight of given volumes of bacterial suspension.

The oxygen consumption and lactic acid production were measured in Ringer solution to which was added the emulsion of bacteria, NaHCO_3 and glucose, the latter in accordance with the requirements of the individual experiments.¹ Anaerobic glycolysis was measured in an atmosphere of 95 per cent N_2 and 5 per cent CO_2 , the bicarbonate concentration being such as to give a pH of approximately 7.8. The aerobic glycolysis was measured in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 . The glucose concentration was 0.05 per cent where not otherwise stated.

TABLE I

Respiration of Pneumococcus Type II in Plain Ringer, and Ringer Solution Containing Glucose

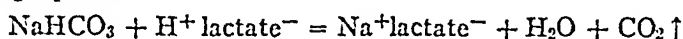
Time in min.	0.5 cc. bacterial emulsion in Ringer solution. No glucose present. c. mm. O_2 consumed	0.5 cc. bacterial emulsion in Ringer solution. Glucose concentration 0.001 per cent c. mm. O_2 consumed	0.5 cc. bacterial emulsion in Ringer solution. Glucose concentration 0.1 per cent c. mm. O_2 consumed
15	4	7	97
15	1	1	40
15	2	2	20
Total O_2 consumed in 45 min.	7	10	157

I. Studies on S Forms of Pneumococcus

Influence of Glucose upon Respiration

To determine whether the oxygen consumption is dependent upon the presence of glucose, the respiration was studied in glucose free

¹ By the method here employed the acid produced is determined manometrically by measuring the CO_2 produced in the presence of NaHCO_3 in accordance with the following equation.



The production of 1 c.mm. of CO_2 indicates that 0.004 mg. of lactic acid has been produced in the fermentation of glucose by the *Pneumococcus*. While the method is not specific for lactic acid, it is known from chemical determinations that lactic acid is the acid produced in the fermentation of glucose by the *Pneumococcus*.

METABOLISM OF S AND R FORMS OF PNEUMOCOCCUS

By PHILIP FINKLE, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research and the Laboratories of the Mount Sinai Hospital, New York)

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The studies of Avery and Heidelberger (1) have shown that in *Pneumococcus* there are present in the capsule certain chemical substances, soluble polysaccharides, which are specific for each of Types I, II, and III. Furthermore, there appears to be a relationship between the virulence of the organism and the presence of the capsule. The so called S form of the *Pneumococcus* is the organism with its capsule intact; and it contains the specific polysaccharide. When the S form becomes transformed into the so called R form of *Pneumococcus*, it has lost its capsule, and as a consequence of this, its soluble polysaccharide, its type specificity, and its virulence as well.

It was considered of interest to study the metabolism of the *Pneumococcus* to ascertain whether variations in metabolism occur in the several types, and whether differences in the virulence are associated with differences in the metabolic activity of the organism. Studies were therefore undertaken of the respiratory and glycolytic function of S and R forms of *Pneumococcus*.

In the first part of this paper are recorded the results of these studies upon the S forms of *Pneumococcus*, Types I, II, and III. In the second part of this paper are given the results of the studies upon the R forms derived from Types I, II, and III.

Methods

For measuring respiration and glycolysis (the splitting of glucose to lactic acid) the method described by Warburg (2), and used by Meyerhof and Finkle (3) in their studies of respiration and glycolysis of lactic acid producing bacteria, was employed.

The pneumococci were grown on phosphate broth of pH 7.5, free of glucose and incubated at 37.5°C. Cultures incubated at periods ranging between 6 and 12 hours were used.

cally equal intensity in pH's from 6.03 to 8.1, it decreases with time, most rapidly at the acid pH 6.03 and least rapidly at pH 7.81.

The minimum oxygen consumption in 1 hour and 10 minutes is 314 mm. at pH 6.03, and the maximum is 610 at pH 7.81. The greatest respiratory activity occurs at that pH which is optimal for growth of the organism.

Respiration and Glycolysis

The classic work of Meyerhof (5) has demonstrated the rôle played by oxygen in the carbohydrate metabolism of muscle. Muscular contraction is followed by production of lactic acid. The same cycle takes place in muscle at rest. Glycolysis, or the splitting of carbohydrate to lactic acid, occurs anaerobically, but the recovery is aerobic.

In the recovery phase lactic acid disappears, being reconverted to glycogen, while only a fraction of the lactic acid (or equivalent) is oxidized completely. The ratio $\frac{\text{Quantity of lactic acid disappearing}}{\text{Equivalent of lactic acid oxidized}}$ has been designated by Meyerhof the

oxidation quotient of lactic acid. In muscle at work this quotient is 4 to 5; in resting muscle it is about 3. In other words, for every mol of lactic acid or its equivalent that is oxidized during the recovery period, 4 to 5 mols of lactic acid disappear, being reconverted to glycogen. In resting muscle, for every mol of lactic acid oxidized, about 3 mols of lactic acid are prevented from being formed.

Warburg and coworkers (6) have shown that a similar relationship exists between oxygen consumption and lactic acid disappearance in the case of the various tissues of warm blooded animals, when those tissues are permitted to respire in the presence of glucose. In the case of those tissues studied the Meyerhof quotient holds true, namely that the utilization of a quantity of oxygen sufficient to burn a given amount of lactic acid (or equivalent) prevents the formation of from three to six times that quantity of lactic acid. This was shown by comparing the aerobic and anaerobic glycolysis, and oxygen respired, by equivalent weights of tissue in a given time interval.

It is interesting that lactic acid production is relatively great in the case of growing cells. In those cells in which growth is orderly—conforming to the pattern of the organism—the respiration is of sufficient magnitude to prevent the appearance of lactic acid in the presence of oxygen. This is not true in the case of pathological growth. Carcinoma is an extreme case in which the appearance of lactic acid is diminished only about 20 per cent in the presence of oxygen. Here the oxygen consumption does not suffice to prevent the appearance of lactic acid.

Meyerhof and Finkle (3) have shown for two varieties of lactic acid producing bacteria, (a) the aerobic *Vibrio metchnikovii*, and (b) the facultative anaerobe, *Bacillus acidificans*, that the formation of lactic

Ringer, and in Ringer to which glucose had been added. The results of a typical experiment are recorded in Table I.

It is evident (Table I) that the presence of glucose results in an enormous (22 fold) increase in respiration. The oxygen consumed in the absence of glucose in 45 minutes is 7 c.mm. as compared to 157 c.mm. in the presence of 0.1 per cent glucose.

The fact that respiration practically disappears in the absence of sugar is interesting in the light of the work of Cole (4). He showed that living pneumococci are able to transform hemoglobin into methemoglobin in the presence of oxygen only if sugar is present. Appar-

TABLE II

Effect of Varying pH's upon Oxygen Consumption of Pneumococcus Type I

Time in min.	0.5 cc. of bacterial emulsion suspended in 1.5 cc. Ringer solution containing 0.05 per cent glucose, and buffered with phosphate buffers			
	pH 6.03	pH 7.01	pH 7.8	pH 8.1
	c. mm. O ₂	c. mm. O ₂	c. mm. O ₂	c. mm. O ₂
10	119	95	107	93
10	81	71	96	93
10	68	85	105	112
10	35	69	87	92
10	11	70	81	81
10	0	70	74	63
10	0	67	60	44
Total in the 70 min.....	314	527	610	585

ently the conditions necessary for respiration and methemoglobin formation are identical.

Effect of Varying pH upon Respiration

The optimum hydrogen ion concentration for growth of the *Pneumococcus* is pH 7.8. The question arises whether the respiration of the organism is influenced by variations in pH, and whether the maximum respiratory activity occurs at the pH which is optimal for growth. Table II gives the results of experiments to determine this point.

It is clear (Table II) that while the respiration begins with practi-

mg. lactic acid) while the aerobic lactic acid production is 58 (0.232 mg. lactic acid). The lactic acid production is suppressed only 62 per cent in the presence of oxygen. The respiration is apparently not sufficient to completely inhibit lactic acid formation aerobically. Hence, *Pneumococcus* Type I has the capacity for aerobic glycolysis.

It is clear from Table IV that while the capacity of *Pneumococcus* Type II to produce lactic acid is practically of the same order as that of Type I, there is no glycolysis in the presence of oxygen. The oxidation quotient is 3.5. The respiration is of sufficient magnitude to completely suppress lactic acid formation. The Type II *Pneumococcus*

TABLE V

Respiration, Anaerobic, and Aerobic Glycolysis of Pneumococcus Type III

Experiment No.	Time in min.	Respiration per mg. bacteria in c.mm. O ₂	Anaerobic glycolysis per mg. bacteria in c.mm. CO ₂	Aerobic glycolysis per mg. bacteria in c.mm. CO ₂	Oxidation quotient
1	30	91	145	0	
2	30	116	161	0	
3	30	65	167	3	
4	30	118	122	4	
Average . . .	30	97	149	—	4.8

cus utilizes 137 c.mm. O₂ per mg. bacteria. The anaerobic lactic acid production, expressed in c.mm. CO₂, is 163. There is no aerobic production of lactic acid.

Pneumococcus Type III (Table V) effects glycolysis anaerobically at the same rate as do Types I and II, but it has no capacity for aerobic glycolysis. The anaerobic glycolysis per mg. bacteria in $\frac{1}{2}$ hour is 149; and 97 c.mm. O₂ is utilized for the same weight and time. The Meyerhof quotient is 4.8. The respiration is of sufficient magnitude to completely suppress lactic acid formation aerobically.

The results shown in Tables III, IV, and V are condensed in Table VI, for the purpose of comparing the respiration and glycolysis of *Pneumococcus* Types I, II, and III.

It is clear (Tables VI) that *Pneumococcus* Type I differs from Types II and III in that it has a high capacity for aerobic glycolysis, about 40 per cent of the anaerobic level. This is due to its diminished

acid is suppressed by oxygen consumption in the ratio expressed by the Meyerhof quotient.

In the studies recorded here it was undertaken to determine to what extent the *Pneumococcus* Types I, II, and III effect glycolysis, how the glycolysis is influenced by the utilization of oxygen, and whether

TABLE III
Respiration, Anaerobic, and Aerobic Glycolysis of Pneumococcus Type I

Experiment No.	Time in min.	Respiration per mg. bacteria in c. mm. O ₂	Anaerobic glycolysis per mg. bacteria in c. mm. CO ₂	Aerobic glycolysis per mg. bacteria in c. mm. CO ₂	Oxidation quotient Meyerhof
1	30	60	113	29	
2	30	37	142	39	
3	30	58	148	70	
4	30	46	173	88	
5	30	69	188	64	
Average....	30	54	153	58	5.4

TABLE IV
Respiration, Anaerobic, and Aerobic Glycolysis of Pneumococcus Type II

Experiment No.	Time in min.	Respiration per mg. bacteria in c. mm. O ₂	Anaerobic glycolysis per mg. bacteria in c. mm. CO ₂	Aerobic glycolysis per mg. bacteria in c. mm. CO ₂	Oxidation quotient
1	30	181	155	2	
2	30	138	204	0	
3	30	130	150	0	
4	30	99	143	5*	
Average....	30	137	163	—	3.5

* These figures for aerobic glycolysis are vanishingly small as compared to glycolysis of 163, and so may be disregarded.

these relationships differ among these 3 types. The results of these studies are condensed in Tables III, IV, and V for *Pneumococcus* Types I, II, and III respectively.

Pneumococcus Type I (Table III) utilizes on the average about 54 c.mm. O₂ per mg. of bacteria per $\frac{1}{2}$ hour. In the same period, the anaerobic lactic acid production, expressed in c.mm. CO₂, is 153 (0.611

For Type I the energy from respiration is 0.259 calory, or three times that from glycolysis. In Type II the respiratory energy is 0.657 calory, almost eight times that for glycolysis. In Type III the energy from respiration is 0.465, six times that from glycolysis. For all three types, the energy derived from respiration is far in excess of that which is derived from anaerobic glycolysis.

If we compare the values for oxygen consumption by the *Pneumococcus* with values found for a human strain (H37) of the tubercle bacillus, and for mammalian tissues, the difference is quite striking. In Table VII, *a*, are condensed the values for respiration obtained in the work recorded here, and the values obtained for the tubercle bacillus by Loebel, Shorr, and Richardson (7), for rat kidney by Warburg, Posener and Negelein (6), and for dog muscle by Richardson, Shorr, and Loebel (8).

TABLE VII, *a*

Comparison of Oxygen Consumption by Pneumococcus Types I, II, and III with That of Tubercle Bacillus and of Mammalian Tissues

Oxygen consumption per mg. dry weight per $\frac{1}{2}$ hr. in c. mm. O ₂					
Pneumococcus			Human (H 37) tubercle bacillus	Rat kidney	Dog muscle
Type I	Type II	Type III			
54	137	97	4	11	1.5

From Table VII, *a*, it is seen that the respiratory metabolism of the *Pneumococcus* is much greater than that of another pathogenic organism, the tubercle bacillus, or of normal mammalian tissue such as kidney and muscle. The respiration of *Pneumococcus* Type I, which is the least intense of the three types studied, is thirteen times as great as that of the tubercle bacillus, while that of Type II, the most intense, is thirty-four times as great. *Pneumococcus* Type II consumes almost 100 times as much oxygen as does dog's muscle.

II. Studies on "R" Forms of Pneumococcus Derived from Types I, II, and III

It becomes of great interest to study the effects upon metabolism (respiration and glycolysis) of removal of the capsular polysaccharide

absence of oxygen has a lactic acid producing capacity of 189, expressed in c.mm. CO₂ (0.757 mg. lactic acid). There is no lactic acid production in the presence of oxygen. The oxidation quotient of Meyerhof is 4.8.

Pneumococcus R III (Table X) utilizes per mg. bacteria in $\frac{1}{2}$ hour 53 c.mm. O₂. In the absence of oxygen the glycolysis expressed in c.mm. CO₂ is 208 (0.832 mg. lactic acid) per mg. bacteria. There is a marked glycolysis aerobically, 61 (0.244 mg. lactic acid) per mg. bacteria. The oxidation is not sufficiently high to completely suppress the production of lactic acid. The latter is depressed only 71 per cent by the respiration.

TABLE X

Respiration, Anaerobic, and Aerobic Glycolysis of Pneumococcus R III

Experiment No.	Time in min.	Respiration per mg. bacteria in c.mm. O ₂	Anaerobic glycolysis per mg. bacteria in c.mm. CO ₂	Aerobic glycolysis per mg. bacteria in c.mm. CO ₂	Depression of lactic acid by O ₂	Oxidation quotient
					<i>per cent</i>	
1	30	49	222	73	67	
2	30	60	204	57	72	
3	30	48	224	52	77	
4	30	56	184	62	66	
Average...	30	53	208	61	71	8.0

TABLE XI

Comparison of Respiration and Glycolysis of Pneumococcus R I, R II, and R III

Pneumococcus	Respiration in c.mm. O ₂	Anaerobic glycolysis in c.mm. CO ₂	Aerobic glycolysis in c.mm. CO ₂	Depression of glycolysis by respiration
				<i>per cent</i>
R I	114	199	0	100
R II	115	189	0	100
R III	53	208	61	71

For the purpose of comparing the respiration and glycolysis of Pneumococcus R I, R II, and R III, the results shown in Tables VIII, IX, and X are condensed in Table XI.

It is apparent (Table XI) that the R form derived from Type III

from the Pneumococcus. In Tables VIII, IX, and X are given the results of such studies upon the R forms derived from Pneumococcus Types I, II, and III respectively.

Table VIII shows that Pneumococcus R I utilizes on the average 114 c.mm. O₂ per mg. dry weight of bacteria per $\frac{1}{2}$ hour. In the

TABLE VIII
*Respiration, Anaerobic, Aerobic Glycolysis of Pneumococcus R I**

Experiment No.	Time in min.	Respiration per mg. dry weight bacteria in c.mm. O ₂	Anaerobic glycolysis per mg. bacteria in c.mm. CO ₂	Aerobic glycolysis per mg. bacteria in c.mm. CO ₂	Oxidation quotient
1	30	132	230	6	
2	30	119	177	0	
3	30	100	210	0	
4	30	104	180	0	
Average....	30	114	199	0	5.4

* The terms R I, R II, and R III, as used in this paper indicate the rough forms of Pneumococcus derived from S strains of Pneumococcus Types I, II, and III, respectively.

TABLE IX
Respiration, Anaerobic, and Aerobic Glycolysis of Pneumococcus R II

Experiment No.	Time in min.	Respiration per mg. bacteria in c.mm. O ₂	Anaerobic glycolysis per mg. bacteria in c.mm. CO ₂	Aerobic glycolysis per mg. bacteria in c.mm. CO ₂	Oxidation quotient
1	30	115	167	0	
2	30	134	208	4	
3	30	113	200	0	
4	30	97	182	0	
Average....	30	115	189	—	4.8

same period the anaerobic lactic acid production expressed in c.mm. CO₂ is 199 (0.796 mg. lactic acid). Aerobically there is no glycolysis. The oxygen consumed suffices to completely suppress the formation of lactic acid. The oxidation quotient of Meyerhof is 5.4.

Table IX shows that Pneumococcus R II consumes 115 c.mm. O₂ per mg. bacteria in $\frac{1}{2}$ hour. This organism, in the same time, in the

respiration, which is 54 c.mm. O₂ per mg. per $\frac{1}{2}$ hour for the Type I, is increased, in the R I to 114, an increase of 110 per cent. For Type III a change of the opposite character takes place. When the S form, which does not possess the capacity for aerobic glycolysis, is converted to the R form, it acquires a marked power to cause glycolysis aerobically. The aerobic glycolysis is almost 30 per cent of the anaerobic glycolysis. The respiration, which for the S form is 97 c.mm. O₂ per mg. per $\frac{1}{2}$ hour is decreased to 53 c.mm. O₂ for the R form, a decrease of 44 per cent.

In the case of Type II, there is no change in the capacity for aerobic glycolysis, when the S form is converted to the R form. Neither form

TABLE XIII

Comparison of Respiration and Glycolysis between S and R Pneumococcus

Respiration per mg. per $\frac{1}{2}$ hr. in c.mm. O ₂			Anaerobic glycolysis per mg. per $\frac{1}{2}$ hr. in c.mm. CO ₂		Aerobic glycolysis per mg. per $\frac{1}{2}$ hr. in c.mm. CO ₂	
Pneumococcus types	S	R	S	R	S	R
I	54	114	153	199	58	0
II	137	115	163	189	0	0
III	97	53	149	208	0	61

effects glycolysis in the presence of oxygen. The respiration for the R form is increased about 20 per cent over that for the S form.

In the case of all three types the anaerobic glycolysis of the R form is higher than that of the S forms by about 25 per cent on the average.

DISCUSSION

For those strains studied, the capacity of the S form of *Pneumococcus* to respire varies with the type. It was found in the study of the S forms that under identical conditions, Type II *Pneumococcus* utilizes 137 c.mm. O₂ per mg. bacteria, *Pneumococcus* III 97 c.mm. O₂, and *Pneumococcus* I 54 c.mm. O₂. The respiration of Type I is 56 per cent of that of Type III which in turn is 71 per cent of that of Type II. Type I differs from the other two types in that it, alone of the three, can effect glycolysis of glucose in the presence of oxygen.

The distinction among the three types was first made on an immuno-

differs from similar forms derived from Types I and II in two respects, first, its capacity for respiration is considerably lower than that of the other two types; and second, it exhibits a marked aerobic glycolysis which is absent in R I and R II. The respiration is apparently too low to suppress aerobic glycolysis.

Although the ability to cause glycolysis is quite constant for all the R forms regardless of type of *Pneumococcus* from which they are derived, there is no such uniformity in respiratory activity. R III possesses about 46 per cent of the respiratory capacity of R I and R II.

Energy Set Free from Respiration and Glycolysis

If we calculate the energy set free in respiration and in glycolysis, we find (Table XII), that for R I and R II there is 0.545 calory derived

TABLE XII
Energy Derived from Respiration and Anaerobic Glycolysis of Pneumococcus R I, R II, and R III

Energy in calories per mg. bacteria per $\frac{1}{2}$ hr. respiration		Energy in calories per mg. bacteria per $\frac{1}{2}$ hr. anaerobic glycolysis
Pneumococcus		
R I	0.545	0.103
R II	0.545	0.098
R III	0.254	0.103

from respiration; more than five times the energy derived from glycolysis, which is 0.10 calory. The energy derived from respiration in the case of R III is 0.254 or about 50 per cent of that found for R I and R II. In all three R forms there is considerably more energy derived from respiration than from glycolysis.

To indicate more clearly the alterations in metabolism which take place on transformation of S to R forms of *Pneumococcus* the results of the foregoing experiments are condensed in Table XIII.

It is interesting to note (Table XIII) that the metabolism of the S forms of Types I and III is altered when they are converted to the R forms. When the S form of Type I, whose capacity for aerobic glycolysis is high (38 per cent of that for anaerobic glycolysis), is converted to the R form, it loses the power of aerobic glycolysis. The

there may be chemical differences among the R forms derived respectively from Types I, II, and III.

The present studies show that these changes from S to R forms are made manifest also by changes in metabolism. Type I, capable of anaerobic activity (that is glycolysis) even in the presence of oxygen, loses this function when converted to the non-virulent form. Type III, on the other hand, incapable of anaerobic activity in the presence of oxygen, assumes this function when it changes from the S to R form. For Type II, alone of the three types, there is no change in capacity for anaerobic activity in oxygen on conversion from S to R form. There is a change in the intensity of respiration and glycolysis, but only in such a way that the respiration is still of sufficient intensity as compared to glycolysis to suppress all aerobic glycolysis.

In these changes in metabolism we have what would appear to be the conversion of cells exhibiting the carcinoma type of metabolism to cells with the normal type of metabolism; and also the reverse of this. Furthermore the newly acquired characteristic is transmitted. *Pneumococcus* Type I, with metabolism of the carcinoma type, when converted to the R form becomes a cell with metabolism of the normal cell type, which transmits this new characteristic. *Pneumococcus* Type III, with metabolism of the normal cell type, when converted to the R form becomes a cell with metabolism of the carcinoma type, and again the new characteristic is transmitted.

The fact that there is a difference between the metabolism of *Pneumococcus* R III and that of the other two R forms would not be consistent with any assumption that the chemical constitution of these cells (*Pneumococcus* R III) is the same as that of R I and R II. The fact that the metabolism is practically the same for the R forms of *Pneumococcus* I and II need not necessarily mean that their chemical structures are alike.

It is interesting to observe that among the strains studied, it was possible to identify the R form derived from S III, by the fact that of all the R forms studied only those derived from S III exhibited aerobic glycolysis.

SUMMARY

In the present paper are given the results of studies on the respiratory and glycolytic metabolism of *Pneumococcus* Types I, II, and III,

logical basis. Subsequently, Avery and Heidelberger (1) found that in the virulent state each type of organism was made up of an immunological species-specific nucleoprotein portion, and a polysaccharide present in the capsule, which is chemically distinct and immunologically specific for each type.

From these studies it is evident that the distinction among the three types is manifested also in the metabolism of each type. Furthermore, it is evident from the fact that Type I effects glycolysis even in the presence of oxygen, that it is a facultative aerobe only, and the possibility suggests itself that the more ideal condition for growth and activity of the organism may be anaerobiosis in the presence of sugar.

The present studies show that when *Pneumococcus* Type I is converted to the rough form, its capacity for respiration increases 110 per cent, with an increase of 92 per cent in anaerobic glycolysis. The capacity for aerobic glycolysis is apparently lost. *Pneumococcus* R I, in the presence of oxygen and glucose, oxidizes the glucose, not splitting any part of it to lactic acid.

In the case of Type II, the conversion of the S to the R form results in a 16 per cent diminution in the capacity for respiration, and a 20 per cent increase in anaerobic glycolysis. R II, as is the case for the S form from which it is derived, does not effect glycolysis aerobically.

When *Pneumococcus* Type III is converted to the R form, the capacity for respiration is diminished 45 per cent, and the capacity for anaerobic glycolysis is increased 40 per cent. The R form derived from Type III assumes the capacity to cause glycolysis aerobically, a function not present in its S form.

When the S forms of *Pneumococcus* become transformed into the R forms they lose their immunological type specificity. The chemical basis for these changes has been elucidated by the work of Avery and Heidelberger. They have shown that the change is accompanied by a loss of the type-specific capsular polysaccharide, the resulting R forms consisting of a nucleoprotein portion,² without apparent type specificity. Although the R forms exhibit common species specificity,

² Recently W. S. Tillett and T. Francis, Jr. (*J. Exp. Med.*, 1930, 52, 561) have shown that there is another fraction, probably a nitrogenous sugar, present in the body of the *Pneumococcus*, common to the R forms derived from the three types. They designate this the Fraction C.

cus Type II consumes over twenty times as much oxygen as does isolated rat kidney tissue, and almost 100 times as much oxygen as does isolated dog muscle.

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and of the R forms derived from these. The metabolism of the S and R forms are compared, and the relationship between changes in virulence, changes in chemical constitution, and changes in metabolism is discussed.

1. There is no respiration in Ringer solution unless sugar is added.
2. The pH (7.8) that is optimal for growth of pneumococcus is also the pH at which the maximum respiration occurs.
3. The intensity of respiration varies with type in the strains used. The respiratory capacity of Type I is 56 per cent of that of Type III, which in turn is 71 per cent of that of Type II.
4. The anaerobic glycolysis is approximately the same for all three groups.
5. Pneumococcus Type I is capable of aerobic glycolysis.
6. Pneumococcus Types II and III do not effect glycolysis aerobically.
7. The energy set free in respiration is considerably greater than that set free in glycolysis.
8. The oxidation quotient for lactic acid is of the same order as found by Meyerhof in muscle and by Warburg for mammalian tissues.
9. The respiratory capacities of Types I and III are changed on conversion of the smooth to the rough form. (a) For Type I the respiration is increased 110 per cent. (b) For Type III the respiration is diminished 45 per cent. (c) For Type II there is only a slight diminution in respiratory activity (16 per cent).
10. The anaerobic glycolysis is increased about 25 per cent on the average for all R forms irrespective of type derivation.
11. Type I on being converted to the R form, loses its capacity for aerobic glycolysis.
12. Type III, on being converted to the R form, gains the capacity for aerobic glycolysis.
13. The oxygen consumption by Pneumococcus compared with that of the human tubercle bacillus and of mammalian tissue, for the same time intervals, weight for weight, is as follows: (a) Pneumococcus Type I consumes thirteen times as much oxygen as does the tubercle bacillus (H37). (b) Pneumococcus Type II consumes thirty-four times as much oxygen as does the tubercle bacillus. (c) Pneumococ-

P

1

2

No. 15-39

No. 15-40 ●

At 189 days, 1.0 cc. HS, Iv.
Anaphylactic death

P, parent

F¹, first generation

F^{II} , etc., succeeding generations

Iv., intravenous

HS, horse serum

Family I.—Animal 15-00 was injected 2 days antepartum. One offspring received its initial injection of horse serum 113 days, the second offspring 189 days after birth. Both died in acute anaphylactic shock. Therefore in this family the animals actively sensitized *in utero* retained their hypersensitiveness for more than 6 months. It follows that active sensitization persists for much longer periods than passive sensitization.² (See chart for Family I.)

CONGENITAL PROTEIN HYPERSENSITIVENESS IN TWO GENERATIONS*†

BY BRET RATNER, M.D., AND HELEN LEE GRUEHL

(From the Departments of Immunology and Pediatrics, University and Bellevue Hospital Medical College, New York University, New York)

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That a fetus can be passively sensitized *in utero* was first pointed out by Rosenau and Anderson¹ in 1906. In such cases, antibodies are transmitted from mother to fetus by way of the placenta. The offspring is sensitive at birth. The sensitization is only transitory, disappearing after 2½ months, as our investigations showed.²

That a fetus can be actively sensitized *in utero*, we recently pointed out.³ Soluble antigen is transmitted from mother to fetus by way of the placenta, and antibodies are developed in the young after birth. This is evidenced by the fact that such young are not hypersensitive at birth, and a definite incubation period must elapse before the presence of anaphylactic intoxication can be demonstrated. Pregnant animals are injected as near term as possible, so that birth may occur before antibodies are established in the maternal circulation. The question now arises whether active sensitization thus induced is transient or persistent.

There are inherent difficulties in the demonstration of active sensitization *in utero*. First, only those families can be used in which birth occurs within a few days after the original parenteral injection of the pregnant animal, that is, before the usual incubation period has elapsed

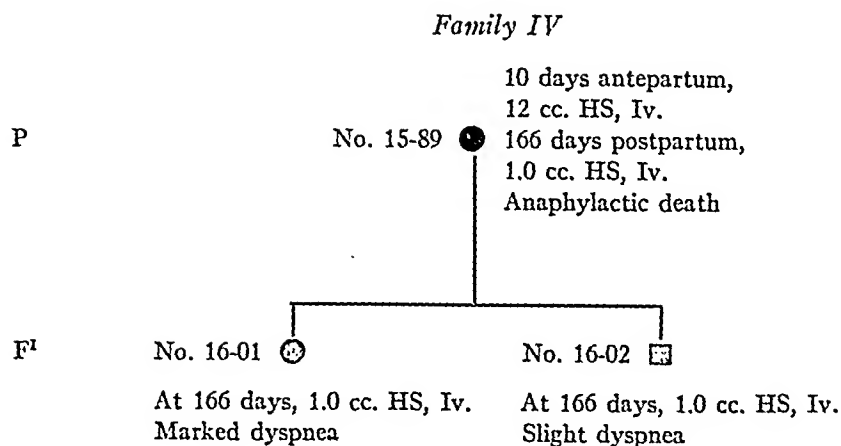
* This work is being carried on under "The Crane Research Fund for the Study of Allergic Diseases in Children."

† Preliminary report, *Proc. Soc. Exp. Biol. and Med.*, 1929, 26, 679.

¹ Rosenau, M. J., and Anderson, J. F., *Bull. Hyg. Lab., U. S. P. H.*, No. 22, 1906, 73.

² Ratner, B., Jackson, H. C., and Gruhl, H. L., *J. Immunol.*, 1927, 14, 291.

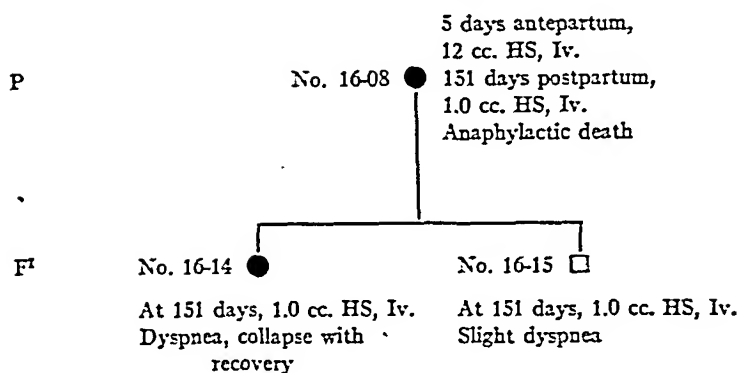
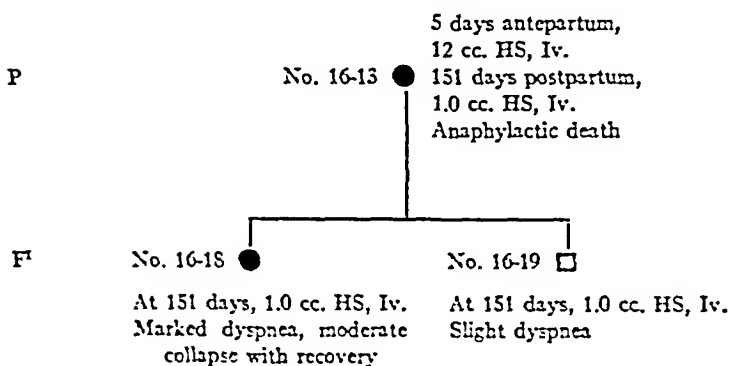
³ Ratner, B., Jackson, H. C., and Gruhl, H. L., *J. Immunol.*, 1927, 14, 303.



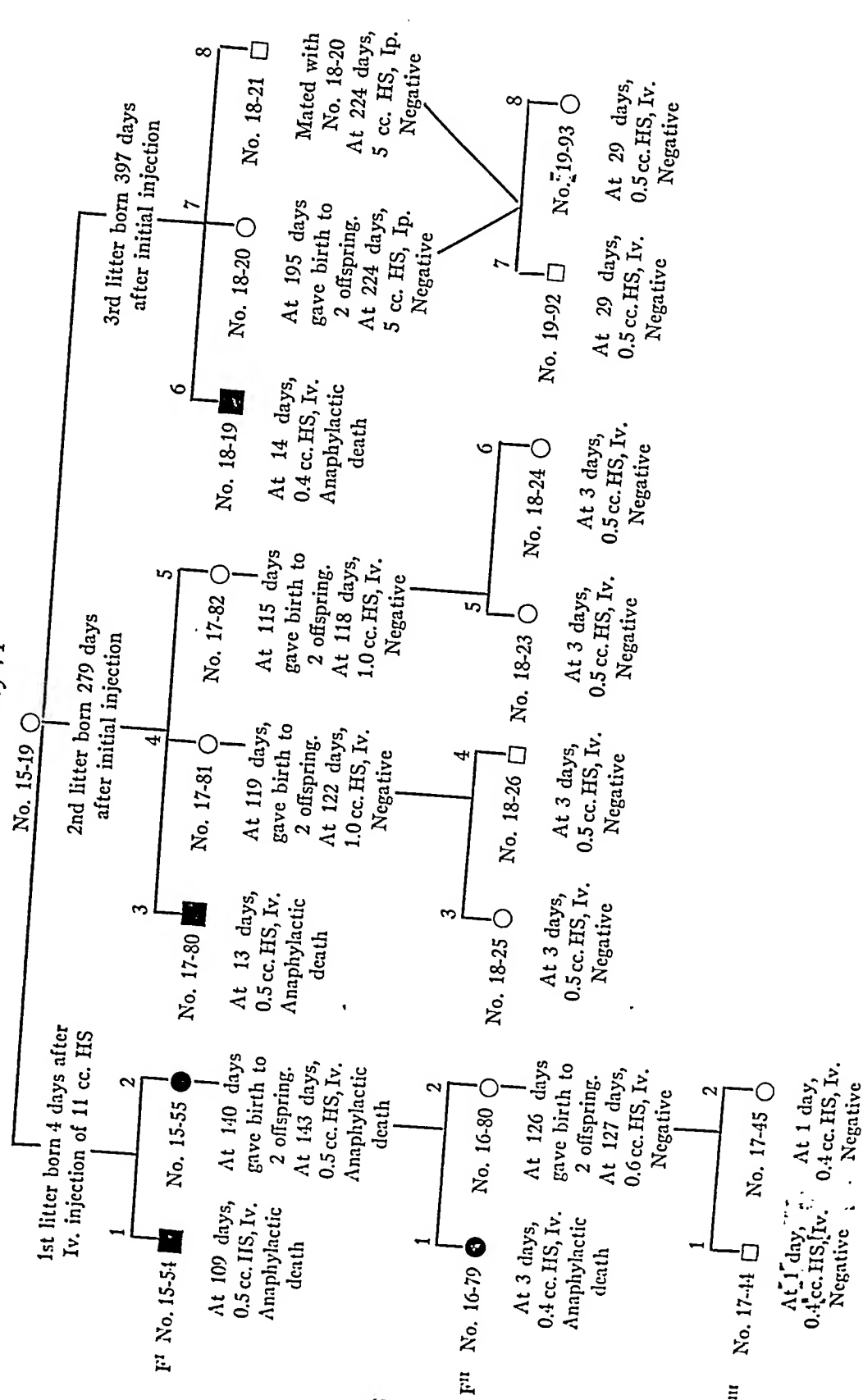
Family IV.—The female was injected 10 days antepartum. Two offspring (F¹) were injected at 166 days; one showed marked dyspnea and the other only slight dyspnea.

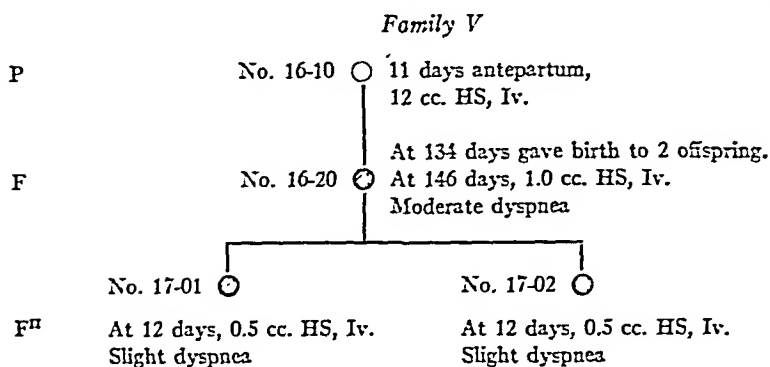
In this experiment it should be noted that the mother died in typical anaphylactic shock when injected the same day as the offspring.

The explanation for the mild reaction of the offspring as compared with that of the mother may be that the sensitivity of the offspring is dual in nature, a result of a combination of transmission of antibodies (passive sensitization) and transmission of antigen (active sensitization). This assumption is made in view of the rather long interval from the date of injection to parturition.

Family II*Family III*

Families II and III.—In both families the pregnant females were injected 5 days antepartum. One animal in each litter (F¹) showed marked anaphylactic intoxication with recovery, and the other showed only mild symptoms on initial injection at 151 days. (See chart for Families II and III.) One can therefore infer that all animals in the same litter do not necessarily acquire the same degree of hypersensitivity.





Family V.—The interval between the initial injection and birth was 11 days. At 134 days the F^I offspring gave birth to two animals (F^{II}). At 12 days these F^{II} animals showed only slight dyspnea when given their initial injection of horse serum. On the same day their mother (F^I generation) was injected and showed moderate symptoms of anaphylaxis with recovery. We have here an example of a slight degree of active sensitization of the F^I generation, with a slight degree of passive sensitization in the F^{II} generation. This may again be accounted for by the long interval between initial injection and parturition as in the case of Family IV.*

* It may be worth while to briefly mention in this connection that when animals are injected with massive doses 5 to 11 days before parturition the antigen appears to be partially neutralized in the fetal circulation; the degree of hypersensitivity is not as profound as in animals actively sensitized, and more nearly satisfies the criteria for passive sensitization. Weil (Weil, R., *J. Med. Research*, 1913, 28, 243) showed that massive doses injected intravenously bring about a very rapid production of antibodies, the incubation period being as short as 8 days. Thus, in Families II and III, in which 5 days elapsed before birth, the offspring may have received antigen plus early formed antibodies, the antibodies neutralizing some of the antigen. As the period increases to 10 and 11 days (Families IV and V), the antigen seems in greatest part to have been neutralized by antibodies, and the hypersensitivity is mild, more nearly resembling that due to passive transmission. In Family V, the female injected 11 days antepartum undoubtedly transmitted a small amount of unneutralized antigen plus antibodies to the F^I generation for the two offspring of the F^{II} generation showed a suggestive state of hypersensitivity when 12 days old; on the same day, the mother (F^I) at the age of 146 days showed a moderate degree of hypersensitivity.

for the establishment of antibodies. Second, as the date of birth of the offspring cannot be predicted within a period of 4 weeks of such calculated term, the number of available families in which active sensitization *in utero* can be determined is extremely limited. We did obtain six such families.

EXPERIMENTAL

Large females (600 to 850 gm.), not previously treated,* were bred with males. The pregnant animals received parenteral injections of horse serum intravenously as near term as possible. We were primarily interested in introducing large quantities of antigen rapidly into the circulation and therefore gave massive doses (10 to 12 cc.). The intraperitoneal route was not adopted because of the possibility of entering the amniotic sac.

Since the purpose of the experiment was to determine the duration of active sensitization, the young were permitted to live under good laboratory conditions for the periods indicated in the protocols, and they were then given an initial injection of horse serum intravenously.

We concerned ourselves only with the transmission through the females: females of successive generations therefore were always bred with normal males.

TABLE I
Passive and Active Sensitization in Utero

	Passive	Active
Sensitizing substance passed from mother (P) to F ^I generation	Antibodies	Antigen
Condition of the F ^I generation at birth	Sensitive	Non-sensitive
Condition of the F ^I generation at 1 mo.	Sensitive	Sensitive
Duration of hypersensitiveness in F ^I generation	2½ mos.	6 mos. or more
Sensitizing substance passed from F ^I to F ^{II} generation	—	Antibodies
Condition of F ^{II} generation at birth	Non-sensitive	Sensitive
Transmission through 1 generation	Positive	Positive
Transmission through 2 generations	Negative	Positive
Transmission through 3 generations	Negative	Negative

A brief statement of the results obtained in each of the six families has been given above.

The accompanying summary (see Table I) compares the criteria for congenital passive and active hypersensitivity.

* These animals had not been previously used in laboratories and were obtained from a reliable source.

Family VI. - First F¹ Litter: A pregnant guinea pig (No. 15-19) was injected 4 days prior to parturition. One offspring (F¹-1) injected at 109 days, died in acute anaphylactic shock, thus showing an active state of hypersensitiveness. The second offspring (F¹-2) when 140 days old, gave birth to two offspring (F¹-1,2). 3 days later the mother (F¹-2) manifested lethal shock on receiving its initial injection of horse serum, thus showing the persistence of active sensitization in that litter (F¹) up to 143 days. On the same day one offspring of the F¹ generation (F¹-1), 3 days old, died upon initial injection of horse serum, indicating a state of passive sensitization in the F¹ generation. {The second animal (F¹-2), when 126 days old, gave birth to two offspring (F¹-1,2) which on the following day were negative when injected for the first time with horse serum. This shows that passive sensitization in the F¹ generation had disappeared.

Second F¹ Litter: The same female (P) was again mated with another normal male and 279 days after the original injection of horse serum gave birth to three animals (F¹-3,4,5). F¹-3 was injected at 13 days and manifested lethal anaphylactic shock, indicating a transmission of antibodies (passive sensitization). F¹-4 at 119 days and F¹-5 at 115 days gave birth to litters of two animals each (F¹-3,4, F¹-4,5). Upon initial injection 3 days after parturition, both mothers and offspring reacted negatively.

F¹ Litter: The same female (P) was again mated with a normal male and 397 days after the original injection gave birth to a third litter of three animals (F¹-6, 7, 8). One of these, F¹-6, when 14 days old, died in acute anaphylactic shock after its initial injection of horse serum. This indicates a passive sensitization from the mother (P).

The other two animals (F¹-7,8) were inbred. When 195 days old, two offspring (F¹-7,8) were born. When 29 days old, F¹-7,8 reacted negatively to the initial injection of horse serum. Both parents also reacted negatively the same day. This corroborates the passive sensitization of the F¹ generation which had disappeared before it could be further transmitted. (See chart for Family VI.)

The above family illustrates in a striking way the differences, in successive generations, of active and passive sensitization. The female F¹ 2, actively sensitized *in utero* through P, gave birth to offspring P¹ which were passively sensitized. The F¹ generation therefore was not hyper-sensitive. In the second F¹ litter the females passively sensitized *in utero*, gave birth to normal offspring. The third F¹ litter, like the second, shows that sensitization of succeeding F¹ litters is passive. This third litter also indicates that inbreeding of the passively sensitized F¹ generation does not appear to augment sensitization in the F¹ generation.

The degree of hypersensitivity, its duration, and its transmissibility appear to be influenced by the time elapsing between the original injection of the parent and parturition.

A pregnant guinea pig receiving a parenteral injection of antigen 2 to 4 days prior to parturition transmits a state of hypersensitivity to two succeeding generations. The sensitization of the F^I generation is due to the passage of antigen. The sensitization of the F^{II} generation is due to the passage of antibodies formed in the F^I generation. This prevents any further transfer of the hypersensitive state.

Though hypersensitivity occurs in two successive generations, the phenomenon is congenital and not hereditary.

We believe that this phenomenon demonstrated in the guinea pig is fundamentally related to the problem of congenital sensitization of the human being.

COMMENT

In our previous work² we showed that passive sensitization *in utero* could not persist much beyond $2\frac{1}{2}$ months. The long period of gestation (70 days) in the guinea pig would prevent an animal, thus passively sensitized, from retaining its hypersensitiveness long enough to transmit it to a succeeding generation.

When a pregnant animal receives a massive injection of antigen 2 to 4 days prior to parturition, her young are not sensitive at birth. Since these offspring subsequently develop a hypersensitiveness, we have postulated that antigen is present in the circulation at birth, and these young develop an active state of hypersensitiveness analogous to that established were they injected intravenously.

Our protocols show that sensitization actively induced *in utero* persists for a longer period than passive sensitization. While we have not determined the farthest limits of the former, our experiments show that circulating antibodies are present when the animal is sufficiently mature to reproduce, and its young are born hypersensitive because antibodies have been transmitted *in utero*. Thus a female sensitized immediately before parturition sensitizes two succeeding generations.

In previous papers^{4,5} it was shown that the human being, like the guinea pig, belongs to the "placentae verae" group in which but a single cell layer separates maternal from fetal circulations. Because of this common anatomical basis for placental permeability, clinical evidence⁶ pointing to sensitization of the human fetus *in utero* was held to be analogous to the experimental situation in the guinea pig.^{2,7}

Specific hypersensitiveness of a human infant need not be placed in the category of true heredity, but of congenital aquisition. The possibility of passage through several generations on the female side is not remote, for reactivation of passive sensitization in succeeding generations might well occur.

CONCLUSIONS

Hypersensitivity actively induced *in utero* is shown to persist for a longer period than passive sensitization.

⁴ Kuttner, A., and Ratner, B., *Am. J. Dis. Child.*, 1923, 25, 413.

⁵ Ratner, B., Jackson, H. C., and Gruehl, H. L., *J. Immunol.*, 1927, 14, 242.

⁶ Ratner, B., *Am. J. Dis. Child.*, 1925, 36, 277.

bulbar conjunctiva was pale, the right cornea normal, the left cornea showed an old trachomatous pannus which occupied a fourth of the corneal surface. Suspensions of excised tissue from the left eye, with the more severe conjunctival changes and the greater number of follicles were used for study. A few inclusion bodies were found.

Case 3.—Severe papillary trachoma of both eyes with follicles in the upper fornix and a few scars in the tarsal conjunctiva of several years' duration in a soldier, 25 years old. The right eye showed a trachomatous ptosis and a fresh pannus 3 mm. wide extending from above with infiltration. The left cornea was normal excepting a few phlyctenular scars in the limbus. Material obtained from the right eye was used in the experiment. No inclusion bodies were found in a single scraping from the epithelium of the upper lid.

Case 4.—Very severe follicular and cicatricial trachoma with slight papillary hypertrophy near the upper end of the tarsal cartilage in a man aged 40. The trachoma was at least 5 years old. Both eyeballs were inflamed and the cornea showed a total pannus with slight keratectasia. Material from the right eye which contained the most follicles, was used for the experiment. Five typical inclusion bodies were found in scrapings from the upper fornix.

Case 5.—Mixed trachoma of both eyes with mucous discharge of 1 year's duration in a male aged 24. Very large follicles were present in the upper fornices, while the upper tarsal conjunctiva was merely thickened by papillary hypertrophy. The bulbar conjunctivae were moderately injected in the periphery. Both corneas showed beginning pannus. The more inflamed eye (left) was used for study. A few small and large inclusion bodies were found in the stained scrapings from the left upper fornix.

Methods

The material used for the inoculation of the media was expressed from the follicles with sterile rolling forceps, and spread directly over the surface of freshly prepared plate media. Other plates were inoculated with a mixture of the expressed material and a suspension in normal saline solution of epithelial cells scraped from the upper palpebral and fornix conjunctiva or with suspensions of excised tissue. From all cases, therefore, the follicle contents and epithelial cells were plated together. The plate media consisted of Huntoon's hormone broth agar containing 1 per cent dextrose and 20 per cent defibrinated horse blood.¹ The inoculated plates were sealed with adhesive tape and placed in tall glass jars containing a little water to prevent drying of the medium.

10 to 20 plates were prepared from the material derived from each patient. The plates were kept at room temperature (20–28°C.) for 9 or 10 days before examination.

At the same time other portions of the same material in dilutions in normal saline solution varying from 1:1 to 1:1000 were inoculated in tall tubes of semi-solid leptospira medium according to the method of Noguchi.¹ Twenty to thirty

STUDIES ON THE ETIOLOGY OF TRACHOMA

BY HOBART A. REIMANN, M.D., AND ARNOLD PILLAT, M.D.

(From the Department of Medicine and the Department of Ophthalmology, Peiping Union Medical College, Peiping, China)

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Bacterium granulosis, *n. sp.*, was recently shown by Noguchi¹ to be the cause of trachoma among the Amerinds of New Mexico. By inoculating special culture media with material obtained from trachomatous conjunctivae he isolated a hitherto unknown bacillus, which when inoculated into the conjunctiva of monkeys produced pathologic changes analogous to trachoma in humans. The disease could be transferred from monkey to monkey and the bacillus could be recovered from the lesions. Lindner,² however, who subsequently examined Noguchi's monkeys believed that the conjunctival lesions were not typical of trachoma but were merely the result of a follicular conjunctivitis (*bindehautfollikulose*).

The prevalence of trachoma among the natives of North China furnished an unusual opportunity for further experimental studies. Attempts were made to confirm Noguchi's work by recovering *B. granulosis* from several typical cases, and to reproduce the disease in monkeys.

Cases Investigated.—Five typical cases were selected for study. A brief clinical description of the cases used follows.

Case 1.—Papillary and follicular (mixed) trachoma of both eyes of about 1 year's duration in a student, aged 20. The bulbar conjunctiva was injected only in the periphery, the left cornea was clear, the right one showed a beginning pannus. Material for study was obtained from the right eye. Stained smears of the scraped off epithelium from the upper fornix of the right eye revealed Prowazek-Halberstadter inclusion bodies, some "cap" forms and some large ones.

Case 2.—Papillary trachoma of the lower and upper lids with many follicles in the upper fornix conjunctiva in a male, 20 years old. Duration 1 year. The

¹ Noguchi, H., *J. Exp. Med.*, 1928, 48, supplement 2, 1.

² Lindner, K., *Arch. Ophth.*, Leipzig, 1929, 122, 391.

and adrenalin was applied three times to the *doubly* everted upper lid. After a short time 0.1 to 0.3 cc. of a heavy suspension of a 4 day growth of *B. granulosis* in normal saline solution was inoculated very superficially into the conjunctival tissue near the canthus externus. Half of the amount was injected from the temporal to the nasal ends along the whole upper fornix. All of the remainder was injected in a small area near the outer canthus so that this region was unusually heavily infected. The fornix conjunctiva as well as the upper tarsal conjunctiva was repeatedly scratched and punctured with the charged needle.

Two of the monkeys, Nos. 8 and 9, were inoculated three times, two, Nos. 1 and 11, were inoculated twice and one received only a single injection. All reinoculations were made in areas previously injected.

Results

Monkeys 8 and 9.—Inoculated three times, May 21, May 29, June 10, 1929. 8 days after the first inoculation there was a slight hyperemia and moderate swelling of the left upper fornix not exceeding the degree of reaction expected from the mechanical trauma. The adjoining conjunctiva of the upper tarsus and of the lower lid and lower fornix conjunctiva and the eyeball conjunctiva were pale and normal. No follicles were visible. After 19 days (June 10), at the time of the third inoculation, Monkey 9 showed a marked swelling of the upper fornix and the upper tarsal conjunctiva. Two follicles, one large, one small, and oblong in shape appeared in the nasal half of the upper fornix. There was no papillary hypertrophy, or thickening of the tarsal conjunctiva.

Monkey 8, 19 days after the first inoculation, developed 3 follicles in the slightly swollen upper fornix, a large one and a smaller one in the inner half and a large one in the outer half. The lid conjunctiva and the conjunctiva in the right eye were normal.

On August 17, 76 days after the first inoculation, the left fornices of both monkeys were somewhat swollen. A regressive follicle was present near the inner angle in Monkey 9. The remainder of the conjunctiva was normal. The structure of the meibomian glands and of the conjunctival blood vessels was normally visible. No traces of scarring or papillary hypertrophy were present.

On September 19, after 118 days, the left upper fornix of Monkey 9 was still swollen and reddened, but the tarsal conjunctiva was normal. Monkey 8 showed slight swelling of the left upper lid. The conjunctiva of the upper fornix was somewhat bluish without any follicles. There were, however, 3 wavy, white, scar-like lines in the upper tarsus. The tarsal conjunctiva and the conjunctiva of the lower lid were normal.

On November 22, 181 days later, Monkey 9 still showed slight hyperemia of the upper fornix conjunctiva, and the tarsal cartilage was not as plainly visible as that

tubes were inoculated with material from each patient. The tubes were kept in the dark, at room temperature for about 2 weeks. At that time transfers were made from the tubes showing growth to other blood agar plates. Many of the tubes remained sterile.

Results

A flora similar to that found by Noguchi grew on the plates, namely staphylococci, xerosis bacilli, sarcinoids and pneumococci. In addition an unusual minute Gram-negative bacillus was isolated from the blood agar plates from 3 of the 5 cases. This organism was apparently identical with the non-pathogenic Gram-negative bacillus described by Noguchi. In one case they were very numerous, but in the other 2 only a few colonies appeared here and there among hundreds of colonies of other bacteria.

From the plates inoculated with the culture from the leptospira medium tubes the same chromogenic Gram-negative bacilli were occasionally recovered. Otherwise the usual organisms mentioned above were found. During the investigation both the tubes and the plates were occasionally overgrown with rapidly growing yeasts and fungi.

Isolation of B. granulosis.—A bacillus corresponding in many respects to *B. granulosis* was recovered from only 1 patient (Case 5).

Only 1 colony of these bacilli was found on 1 of the 12 blood agar plates inoculated with a mixture of follicle contents and scraped off epithelial cells. The rest of the colonies including several of those of the chromogenic Gram-negative bacillus consisted of the variety usually encountered. The characteristics of the new Gram-negative bacillus corresponded closely with the morphologic description of *B. granulosis*. It was non-chromogenic, and produced characteristic viscid, grayish, translucent colonies on blood agar in 48 hours at room temperature. Growth at 37°C. was retarded and scanty. No growth at all occurred on plain agar or in broth at any temperature. Experiments to determine motility were unsatisfactory. Growth in leptospira medium, however, was poor and occasionally no growth at all occurred at either 28 or 37°C. Bacilli of this variety were never encountered on the platings made from the leptospira medium tubes inoculated with trachomatous material.

Experiments on Monkeys

Method of Inoculation.—The left eye was used in each instance, the right serving as a control. The upper fornix conjunctiva of 5 monkeys (*Macacus sinensis*) was inoculated. A 4 per cent solution of cocaine

man. The swelling and hyperemia of the conjunctiva seen in the first few weeks after the inoculation of a heavy suspension of bacteria and the mechanical trauma of the needle did not exceed the degree of reaction to be expected after such a procedure. The reaction faded in the succeeding weeks but did not vanish entirely. The few sharply defined, oval superficial follicles found in 3 of the monkeys, probably have but little significance, since their number did not increase. Furthermore, the velvety unevenness, papillary hypertrophy and obliteration of the meibomian glands and of blood vessels of the conjunctiva commonly observed in human trachoma did not appear.

Nevertheless there are 3 important points to consider:

1. The conjunctival hyperemia in 4 monkeys persisted as long as 6 months. Whether this prolonged reaction was the result of the trauma of repeated inoculation of bacteria (foreign body irritation) or whether it indicated an unusual reaction of beginning trachoma is at present difficult to say.

2. The presence of unusual white lines in the inoculated lids of 3 monkeys: The lines were distinct from the whitish meibomian glands found in *Macacus sinensis*. They appeared to lie in the inner strata of tarsal cartilage or between the conjunctiva and the tarsal cartilage. 3 months after their appearance the white lines had nearly disappeared. Their nature is at present obscure. They may have been scars or zones of infiltration or demarcation delimiting normal and pathologic tissue. Their early disappearance indicates the latter probability.

3. The progressive shrinkage of the tarsal cartilage found in 2 monkeys, Nos. 9 and 11. This was further accentuated by an incurving of the otherwise plane cartilage found in *M. sinensis*. Here again it is difficult to judge whether this scarring was the result of the traumatism of inoculation of bacteria or whether it was actually a trachomatous lesion. It appeared, however, to be a progressive lesion caused by a chronic irritation. We wish to emphasize the latter probability since traumatic scarring would have appeared earlier than 5 months after first inoculation and would probably not have progressed.

Whether one is justified in correlating these facts with the clinical picture of human trachoma only further work can show. There was, indeed, a shrinkage of the tarsal cartilage, apparently produced by the

of the right eye. The cartilage was curved inward slightly. Curiously, the tarsal conjunctiva appeared to be normal and the long white stripes of the meibomian glands were plainly visible through it. There was still redness of the upper fornix in Monkey 8.

On December 28, 217 days after the first inoculation, the first definite and significant changes were observed. Although the upper fornix conjunctiva of Monkey 9 was still somewhat red and swollen, but with follicles, the upper tarsal cartilage was definitely curved inwards as if shrunken. Its horizontal diameter was 1 mm., and the vertical diameter 0.75 mm. shorter than those of the right eye. The adjacent conjunctiva was slightly injected but showed no trace of papillary hypertrophy or of scaling. The right eye was normal.

The 3 white lines observed in Monkey 9 in September gradually faded until only one was visible. The conjunctiva was normal.

Monkeys 1 and 11.—Inoculated twice at an interval of 11 days. At the time of reinoculation, only Monkey 11 showed slight swelling, and hyperemia of the upper fornix conjunctiva with 1 small follicle near the external canthus.

Upon examination 68 days later there was a mild bilateral angular blepharoconjunctivitis in Monkey 1. The traumatic irritation observed previously in the left upper fornix had disappeared. In Monkey 11 the left upper fornix was redder than the right one, and the follicle had disappeared. The rest of the conjunctiva was pale and normal.

110 days after inoculation the left eye of Monkey 1 showed an angular blepharitis, the left upper fornix was still somewhat swollen but the tarsal conjunctiva was normal. The left upper fornix of Monkey 11 was swollen and hyperemic but devoid of follicles. Here again, in the otherwise pale conjunctiva of the upper lid, was a curved white line about 5 mm. long in the outer half of the tarsal cartilage.

173 days after the first infection the tarsal conjunctiva of Monkey 1 was pale, and the cartilage normal. The conjunctiva of the fornix was still hyperemic, swollen and there were 3 follicles near the outer canthus. There was an angular blepharitis without discharge. By this time the upper tarsal cartilage of the left eye had become definitely smaller than that of the right one (about 1 mm. in each dimension) and curved inward. The white line previously noted had vanished. The overlying conjunctiva was thin, pale and transparent. The upper fornix conjunctiva of both eyes was slightly reddened. Otherwise there were no changes. After 209 days the same characteristics were present.

Monkey 12.—This monkey which was inoculated only once merely showed moderate swelling and hyperemia of the conjunctiva of the injected lid after 67 days. Soon after, the animal died from other causes.

DISCUSSION (Pillat)

Although definite pathologic changes have been induced in the eyelids of *Macacus sinensis* by the inoculation of *B. granulosis* Noruchi it is clear that the lesions do not resemble trachoma as it is seen in

thermore, the monkeys we used (*M. sinensis*) were of a species hitherto untested. It is possible that this species reacts to inoculations of *B. granulosis* less typically than do *rhesus* monkeys and chimpanzees.

SUMMARY

A bacillus which corresponds closely with the description of *B. granulosis* Noguchi was isolated from 1 of 5 typical cases of trachoma in Chinese. Inoculation of suspensions of this bacillus into the eyelids of *M. sinensis* monkeys produced scarring and contraction of the tarsal cartilages with the development of a few conjunctival follicles in 2 out of 5 animals. The possible relations of the observed lesions with human trachoma have been discussed.

subconjunctival inoculation of *B. granulosis* Noguchi, which first appeared several months after the first infection, but no trachomatous changes of conjunctival tissue ever appeared. There are, however, 2 types of trachoma in man, mild, which often heals without visible scarring, and severe, in which the tarsal cartilage is profoundly affected by extensive scar formation.

Prowazek-Halberstädter inclusion bodies are commonly found in large numbers in early trachoma when the conjunctiva is chiefly involved. The further extension of the infection to the tarsal cartilage, the bulbar conjunctiva and the cornea can hardly be attributed to inclusion body invasion since these bodies are never found in subconjunctival tissue or in the tarsal cartilage. There are other infectious eye diseases (inclusion blennorrhea and swimmer's conjunctivitis) in which similar if not identical epithelial inclusions occur. During the early period of these diseases, inclusions are usually found in great numbers. The diseases, however, are of short duration, follicles are formed but the cornea is not involved and no scars form. The inclusion bodies disappear with recovery.

A consideration of these facts together with our experimental results in monkeys suggests the possibility that human trachoma may be in most instances a double infection. In other words, there may be first a superficial conjunctival infection associated with inclusion bodies or Lindner's "initial" bodies and second, a deep infection of the subepithelial layers probably caused by *B. granulosis* Noguchi. The question of a symbiosis between *B. granulosis* and the agent producing inclusion bodies must await further study.

Our inability to isolate *B. granulosis* from as great a proportion of cases of trachoma as Noguchi may be due to variation from the methods used by him, or to differences in the composition of the culture media. He used hormone broth which had been neutralized and many carbohydrates in his blood agar plates. Cocaine was used for anesthetizing the conjunctiva of monkeys before inoculation and it has since been shown that cocaine has a destructive action on *B. granulosis*. However, the cocaine solution was only applied to the surface while the cultures were injected into the conjunctival tissue. Of necessity a culture from a single colony was used by us for inoculation whereas Noguchi combined cultures from several colonies. Fur-

Materials and Methods

The experimental results used in this analysis were those obtained from the weekly blood examinations on 45 normal rabbits distributed in five groups as follows:

Group	Number of rabbits	Number of examinations	Number of counts	First count	Last count
I	10	35	350	Oct. 24, 1927	June 20, 1928
II	10	13	130	Mar. 29, 1928	June 19, 1928
III	10	8	80	Sept. 20, 1928	Nov. 22, 1928
IV	10	29	283	Nov. 27, 1928	June 18, 1929
V	5	26	130	Dec. 29, 1928	June 21, 1929
	45	111	973		

The conduct of the experiments has already been described (2). The rabbits were young adult male animals approximately 8 months of age and the majority represented browns, greys, and Flemish crosses. At the onset of the experiments all animals were apparently free from disease, but three of Group I and six of Group IV developed clinical snuffles while under observation; of the latter group, two died during the course of the experiment and two others soon after its termination. Groups II and III observed for short periods and Group V were clinically free from intercurrent disease. Two cases of ear canker developed in Group I and there were four cases in Group IV. For the most part, however, the general condition of the rabbits was excellent, and a gain in body weight was the rule.

The method employed in the examination of the blood has been described (2). The differential counts were made with the neutral red supravital technique; the red and white cell counts were made with standardized pipettes, and the hemoglobin content was determined by the Newcomer method. The examinations were carried out on the same day of consecutive weeks with the exception of Group III in which the first four counts were made at irregular intervals.

The results are analyzed upon the basis of the weekly mean group values of the various classes of cells and of the hemoglobin content; these values have already been published (2, 3, 4, 5). The existence of technical errors in the blood examinations and the occurrence of individual physiological as well as possible pathological states which might be reflected in variations of the blood constituents determined the employment of mean values. In the present analysis, both smoothed and unsmoothed values were used; the formula employed for smoothing

$$\text{was } \frac{a + 2b + c}{4}.$$

The measure of the degree of relationship which existed between any two blood constituents was determined by the use of correlation coefficients. Simple zero

STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

VI. BLOOD CELL RELATIONSHIPS IN GROUPS OF NORMAL RABBITS
WITH RESPECT TO TIME

BY ALBERT E. CASEY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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In previous papers (1, 2, 3, 4, 5) repeated observations were reported on the cells and the hemoglobin content of the peripheral blood of normal rabbits, and attention was drawn both to the numerical variations of these elements and to the nature of the variations. The results which were analyzed from the standpoint of the weekly mean values of each animal group examined over long periods of time, clearly demonstrated that the cells and the hemoglobin fluctuate to a greater or less degree. These results were obtained under as uniform experimental conditions as was possible with the usual laboratory equipment. In many instances, the differences in cell level were found to be statistically significant and the rate of change was often of an orderly nature. It was also shown that the trends in two parallel groups of animals examined over the same months were on the whole similar both as regards time and direction, although the general cell levels themselves were different.

The analysis showed further that variations in the levels of certain classes of cells were accompanied by similar or opposite variations in other classes. Between still other cell types, there was no apparent relationship.

In order to determine the significance of these trends and fluctuations from the standpoint of the association or lack of association in the movements of the various cell levels and of the hemoglobin content, a statistical analysis of the material has been made, the results of which are contained in the present paper.

That $R = 1.00$ when the white blood cells are the dependent variable, is explained by the fact that the value of the white blood cells is equal to the sum of the values for the various classes of white cells, consequently making an excellent check column.

In order to determine to what extent the total shift in a given type of cell was accompanied by or associated with similar shifts in other cells, multiple correlation coefficients were calculated for the eight classes of blood elements studied. Multiple correlations were also calculated upon the simple correlation coefficients of Groups I, II, IV, and V to check the validity of the combined values.

Upon the assumption that the simple correlation coefficients combined for all five groups were representative of our experience with the various intercellular relationships from the standpoint of time trends in group values, it was desirable to know whether these various relationships were independent of each other and to what degree. To this end, partial correlations of various orders up to the fifth were made upon each of the 21 possible relationships (6); the relationships of the total white blood cells to the various classes of white cells could not be included since these values make up the total figure. As a check on the partial correlations of the combined coefficients, partial correlations were made upon the simple coefficients for the individual groups and no striking differences were noted.

The group means were also analyzed from the standpoint of the relationship between short fluctuations holding the long time trends constant. Theoretically, the variations of two classes of cells might be related in trend over longer periods of time but might be unrelated or inversely related as regards short time fluctuations, assuming different causes for the trend and the fluctuation. The variate difference correlation method was employed in analyzing the results along these lines (8, 9, 10). Successive differences to the sixth were determined upon the unsmoothed weekly group means for the various blood elements in the five animal groups, and simple correlations were then calculated upon the sixth differences for the 28 possible combinations. It was found that the significance of the coefficients could be approximately determined for the sixth difference on these short series by making $n = n' - 2 - 6$ and calculating the probability as if it were a simple zero order coefficient or by making it twice the value for the simple zero order coefficients. The standard of comparison was the method for the probable error developed by Anderson (11). Again, in order to check the accuracy of the coefficients listed, multiple R was calculated for these variate sixth difference coefficients with the total white blood cells as the dependent variable and the various classes of white cells as the independent variables; it was found to be equal to unity ($R = 1.00$). In addition, these coefficients were averaged for all five groups by the method of transformed correlations and the multiple correlation coefficient calculated upon these averaged values was still equal to unity. The homogeneity or the lack of it was determined by the use of transformed correlations, making $n = n' - 3 - 6$.

Partial fifth order correlation coefficients were calculated upon the variate

order correlation coefficients were calculated upon the smoothed weekly means for each of the five animal groups, using the formula $R_{xy} = \frac{S_{xy} - M_x S_y}{N s_x s_y}$ (6). The number of blood elements followed, including the total white blood cells, was eight, thus offering possibilities for 28 coefficients. The significance of each coefficient has here been expressed in terms of probability (7). Any result which might occur from the random association of two variables to the extent of five or more times per hundred chances ($P = .05$) has not been interpreted as significant. A value has been considered as probably significant when the chances of its occurring by accident or random association are between two and five per hundred chances ($P = .02 - .05$). The designation of significant has been assigned a value when the chances of its occurring by accidental relation between two variables is one or less than one per hundred chances ($P = .01$).

Significant differences in the correlation coefficients between the various animal groups were determined by the method of "z," that is, the method of transformed correlations. Published tables are available for these calculations (7). Unless the difference between two transformed correlations was greater than twice its standard error, the two correlation coefficients were assumed to be from the same or similar material. When the coefficients for all five groups were found not to be significantly different from each other, they were said to be homogeneous. If the simple zero order coefficients for the groups examined 26 to 35 weeks, that is, Groups I, IV, and V, were not significantly different from each other, they were considered as probably homogeneous, regardless of the values for Groups II and III, the observations on which were too few to be of much statistical importance. If any significant difference existed between the coefficients of Groups I, IV, V, the coefficients listed for that relationship were said to be non-homogeneous.

By this same method of transformed correlations, homogeneous coefficients were averaged so as to give a general combined expression for any given relationship (7). Such a combined expression was calculated for each of the 28 simple relationships both including and excluding Groups II and III, and it was found that the inclusion or exclusion of these shorter groups made no significant difference in the results. The calculation was carried further by combining the coefficients of correlation for all five groups regardless of their homogeneity in order to compare the averaged relationships with each other on a common basis. Any interpretation of these results, however, must necessarily be made with caution and with reference to the values for individual groups.

The technical accuracy of the calculations was checked through the use of the multiple correlation coefficient (6). The averaged correlation coefficients for all five groups were used as the basis, and a multiple correlation coefficient was calculated in which the total white blood cells was the dependent variable, and the neutrophils, the eosinophiles, the basophiles, the lymphocytes, and the monocytes, the independent variables. The multiple correlation coefficient was found to be equal to unity ($R = 1.0$), signifying that no errors in calculation had been made.

TABLE I

Simple Zero Order Correlations upon the Smoothed Weekly Means of Blood Constituents in Normal Rabbits

ents in Normal Rabbits

Means of Blood Consti-

Animal group	Simple values					Combined values		
	I	II	III	IV	V	I, IV, V	I, II, III, IV, V	
	n'	35	13	8	29	26	84	99
WN		+ .867 S	+ .913 S	+ .860 S	+ .943 S	+ .929 S	+ .915 S	+ .913 S
WB		+ .686 S	+ .743 S	-.240	-.074	+ .012	+ .302 S	+ .336 S
WE		-.173	+ .174	-.137	+ .465 PS	+ .546 S	+ .260 PS	+ .232 PS
WL		+ .918 S	+ .929 S	-.123	+ .454 PS	+ .891 S	+ .829 S	+ .823 S
WM		+ .406 PS	+ .642 PS	-.340	+ .647 S	+ .659 S	+ .567 S	+ .540 S
WR		+ .555 S	+ .676 PS	-.377	-.253	+ .283	+ .252 PS	+ .276 S
WH		+ .819 S	-.161	+ .191	-.094	+ .527 S	+ .531 S	+ .456 S
RH		+ .227	-.222	-.081	+ .474 S	+ .497 S	+ .390 S	+ .309 S
RN		+ .380 PS	+ .398	-.592	-.355	+ .220	+ .102	+ .094
RB		+ .503 S	+ .936 S	+ .367	+ .452 PS	-.015	+ .354 S	+ .470 S
RE		-.166	-.393	-.065	+ .017	+ .105	-.031	-.073
RL		+ .527 S	+ .767 S	+ .512	+ .132	+ .350	+ .360 S	+ .425 S
RM		+ .523 S	+ .353	+ .929 S	-.279	+ .110	+ .167	+ .261 S
HN		+ .908 S	-.031	+ .116	-.068	+ .564 S	+ .641 S	+ .567 S
HB		+ .482 S	-.339	+ .048	-.078	+ .027	+ .188	+ .125
HE		-.150	-.249	+ .009	-.264	+ .096	-.119	-.126
HL		+ .638 S	+ .025	+ .059	-.032	+ .487 PS	+ .414 S	+ .360 S
HM		+ .221	-.273	-.058	-.255	+ .067	+ .023	-.013
NB		+ .428 PS	+ .467	-.597	-.208	+ .009	+ .115	+ .114
NE		-.236	+ .481	-.201	+ .355	+ .447 PS	+ .156	+ .175
NL		+ .645 S	+ .752 S	-.550	+ .183	+ .696 S	+ .541 S	+ .523 S
NM		+ .250	+ .607 PS	-.632	+ .588 S	+ .560 S	+ .460 S	+ .426 S
BE		-.020	-.339	+ .406	+ .322	+ .193	+ .153	+ .116
BL		+ .658 S	+ .770 S	+ .440	-.143	-.118	+ .228 PS	+ .316 S
BM		+ .203	+ .465	+ .626	-.099	-.117	+ .016	-.077
EL		-.276	-.040	-.146	+ .054	+ .415 PS	+ .031	+ .014
EM		+ .308	+ .149	+ .071	+ .442 PS	+ .449 PS	+ .398 S	+ .359 S
LM		+ .279	+ .497	+ .423	+ .010	+ .494 PS	+ .264 PS	+ .299 S

In this and all other tables the

In this and all other tables the following symbols are used:

n' = Number of observations.

r = Coefficient of correlation.

S = Significant.

PS = Probably significant.

W = Total white blood cells.

N = Neutrophiles.

B = Basophiles.

E = Eosinophiles.

L = Lymphocytes.

M = Monocytes.

R = Total red blood cells.

H = Hemoglobin.

difference coefficients averaged by the method of transformed correlations. No precedent can be given for such a procedure, but if the variate difference values represent any true relationships whatsoever, then such relationships should be susceptible to partial and multiple correlation. The significance was calculated by assuming that for any simple combined variate sixth difference coefficient $r = 67$ where the original number of weekly group means was 111. The significance of the partial fifth order coefficient was read in terms of probability making $n = 67 - 5$.

Recapitulation

This statistical study of the trends and variations in the levels of the cells and the hemoglobin content in the peripheral blood of normal rabbits was carried out for the purpose of determining whether an association or lack of association obtains between the observed fluctuations of these elements. The values employed for the analysis were the unsmoothed weekly means obtained from the observations on five groups of rabbits examined over long periods of time; these values have been published in previous papers (2, 3, 4, 5). The results are contained in a series of tables and charts.

Table I gives the simple trend correlation coefficients for each of the 28 simple relationships of the various cells and of the hemoglobin content. The combined coefficients for the three groups followed for the longest times, that is, Groups I, IV, and V, and for all five groups are listed in the last two columns at the right side of the table.

Table II gives the variate sixth difference correlation coefficients for the 28 simple relationships in four animal groups; Group III contained too few observations to be treated by this method, and although Group II was little better in this respect, it has been included to show the limits of the method. The right hand column of the table contains the coefficients of correlation averaged by the method of "z" for Groups I, IV, V, and also for Groups I, II, IV, and V.

Table III contains a summary of the averaged coefficients obtained by the method of the variate sixth difference and by that of simple trend correlations. Partial correlations of the fifth order (holding all factors constant except total white blood cells) upon the combined coefficients obtained by each of these two methods are given in the column to the right of the zero order coefficients.

Table IV summarizes the multiple correlation coefficients obtained from the combined values by both the variate difference and the simple trend correlation methods. The values for both R and R^2 are given.

The curves of Text-figs. 1, 2, 3, and 4 represent the percentage deviations of the

TABLE III

Summary of Analyses of Combined Correlation Coefficients (Method of "Z" Applied to Five Groups of Normal Animals)

Relation	Simple zero order correlation	Partial 5th order correlation	Variate 6th difference correlation	Variate 6th difference, 5th order partial
WN	+.9125 S		+.7630 S	
WB	+.3360 S		+.2800 PS	
WE	+.2317 PS		+.0990	
WL	+.8225 S		+.4996 S	
WM	+.5395 S		+.3720 S	
WR	+.2755 S		+.3214 PS	
WH	+.4560 S		-.1215	
RH	+.3093 S	+.4349 S	+.3065 S	+.5202 S
RN	+.0937	-.4030 S	+.2537 PS	+.4412 S
RB	+.4696 S	+.4281 S	+.5579 S	+.6665 S
RE	-.0726	-.1514	-.0005	-.4050 S
RL	+.4249 S	+.4148 S	+.2848 PS	+.0917
RM	+.2605 S	+.4425 S	+.2440 PS	+.4662 S
HN	+.5670 S	+.6334 S	-.2120	-.3065 PS
HB	+.1254	-.1185	+.1276	-.2241
HE	-.1264	-.1007	-.1197	+.1140
HL	+.3600 S	-.0555	+.0706	-.0903
HM	+.0125	-.3939 S	-.4496 S	-.5230 S
NB	+.1140	+.0857	-.1741	-.2807 PS
NE	+.1746	+.1072	-.1139	+.0030
NL	+.5228 S	+.4215 S	-.2415 PS	-.2594 PS
NM	+.4256 S	+.4667 S	+.3331 S	+.0598
BE	+.1155	+.2006 PS	+.4683 S	+.6347 S
BL	+.3156 S	+.1293	+.3908 S	+.2800 PS
BM	+.0771	-.2122 PS	+.0634	-.3058 PS
EL	+.0144	-.1045	-.0529	-.2612 PS
EM	+.3585 S	+.3114 S	+.2564 PS	+.3840 S
LM	+.2989 S	+.0086	-.0142	+.0454

TABLE IV

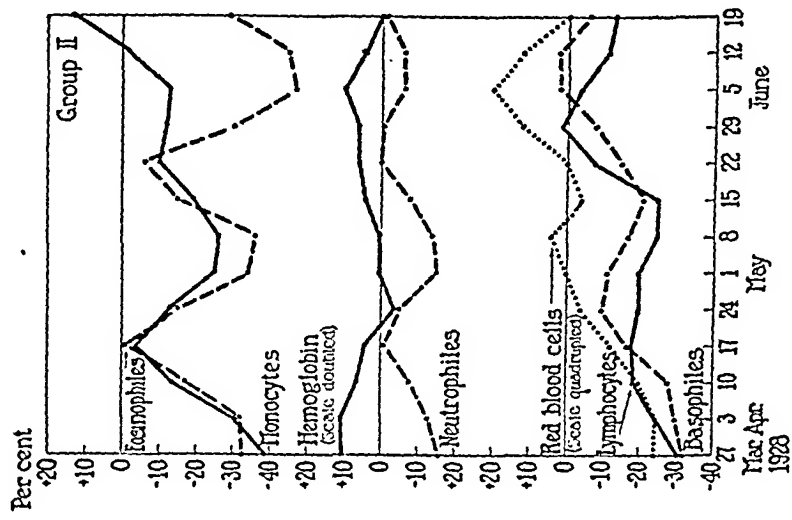
Multiple Correlation Coefficients upon Combined Simple and Variate 6th Difference (Zero Order) Coefficients (Groups I, II, III, IV, and V)

Class of cell	Zero simple correlation		Zero 6th difference correlation	
	R ²	R	R ²	R
Red blood cells.....	.480	.690	.682	.826
Hemoglobin.....	.510	.716	.456	.676
White blood cells.....	1.014	1.007	1.142	1.069
Neutrophiles.....	.640	.802	.370	.609
Basophiles.....	.290	.534	.679	.824
Eosinophiles.....	.210	.453	.470	.686
Lymphocytes.....	.430	.656	.278	.527
Monocytes.....	.410	.642	.474	.689

TABLE II

Variable (6th) Difference Correlations upon the Unsmoothed Weekly Means of Blood Constituents in Normal Rabbits

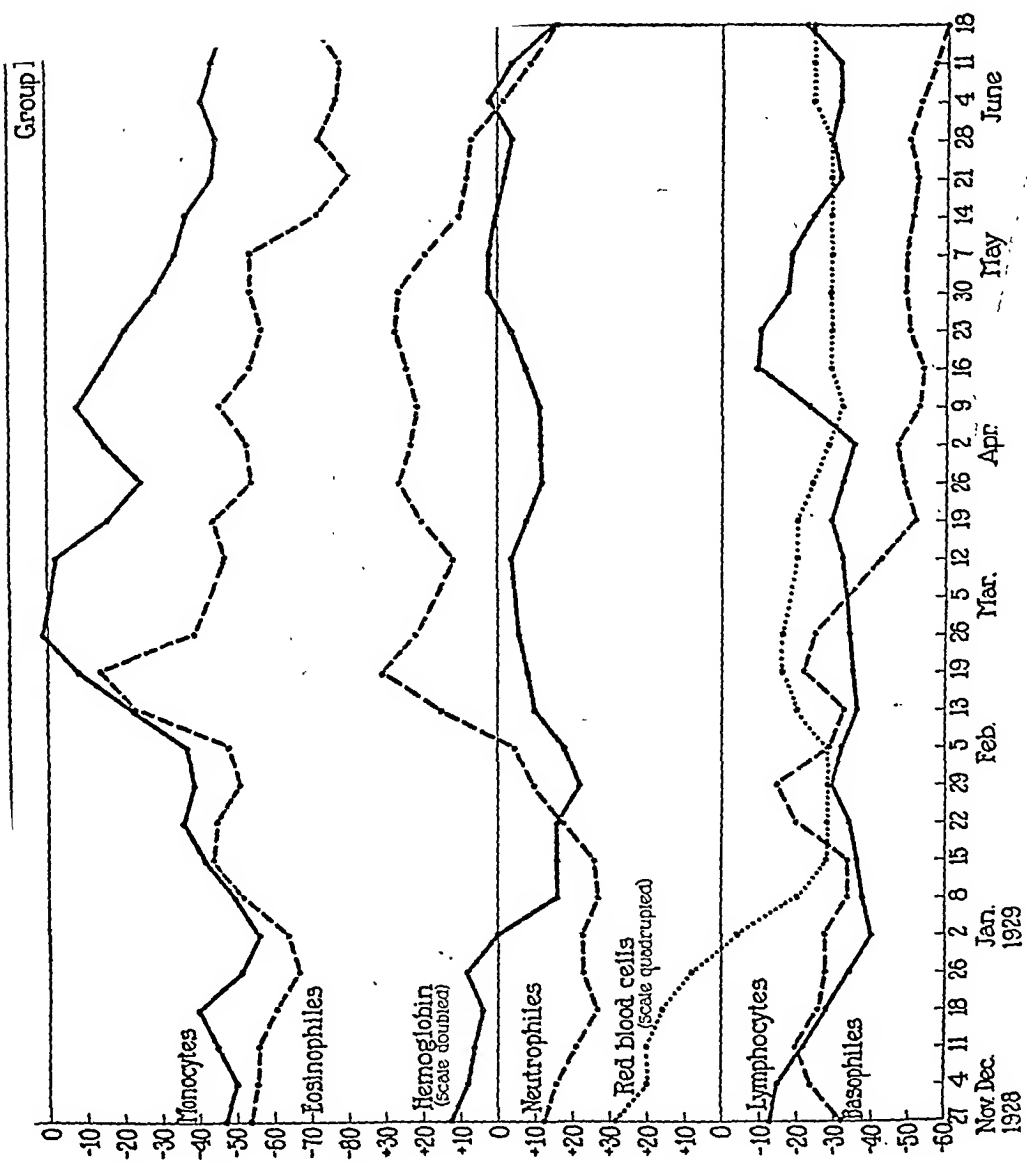
Animal group	Simple values				Combined values	
	I	II	IV	V	I, IV, V	I, II, IV, V
<i>n'</i>	35	13	29	26	65	70
WN	+.448 PS	+.948 S	+.890 S	+.807 S	+.745 S	+.763 S
WB	-.135	+.968 S	+.637 S	-.029	+.173	+.280 PS
WE	+.212	+.405	+.574 S	-.669 S	+.078	+.099
WL	+.042	+.929 S	+.633 S	+.684 S	+.445 S	+.500 S
WM	+.085	+.960 S	+.397	+.427	+.284	+.372 S
WR	-.387 PS	+.986 S	+.774 S	+.138	+.195	+.321 S
WH	-.091	+.549	-.471 PS	+.114	-.167	-.122
RH	+.468 S	+.499	-.142	+.471 PS	+.293 PS	+.307 PS
RN	-.367 PS	+.969 S	+.832 S	-.279	+.143	+.254 PS
RB	+.454 PS	+.942 S	+.649 S	+.387	+.503 S	+.558 S
RE	-.195	+.329	+.155	+.034	-.022	-.001
RL	+.096	+.874 S	+.310	+.304	+.222	+.285 PS
RM	+.036	+.917 S	+.251	+.249	+.164	+.244 PS
HN	-.360 PS	+.326	-.101	-.227	-.245	-.212
HB	+.312	+.728	-.371	+.247	+.077	+.128
HE	-.179	+.879 S	-.168	-.310	-.212	-.120
HL	+.359 PS	+.743	-.688 S	+.441	+.014	+.071
HM	-.608 S	+.506	-.530 PS	-.255	-.500 S	-.450 S
NB	-.762 S	+.851 PS	+.637 S	-.331	-.261	-.174
NE	-.237	+.113	+.451 PS	-.590 S	-.128	-.114
NL	-.817 S	+.764 PS	+.268	+.218	-.315 PS	-.242 PS
NM	+.232	+.857 PS	+.141	+.490 PS	+.280 PS	+.333 S
BE	+.487 S	+.612	+.347	+.532 PS	+.459 S	+.468 S
BL	+.567 S	+.973 S	+.188	-.090	+.293 PS	+.391 S
BM	-.083	+.935 S	-.099	-.011	-.041	+.043
EL	+.160	+.672	+.243	-.735 S	-.105	-.053
EM	+.081	+.477	+.533 PS	+.095	+.242	+.256 PS
LM	-.364 PS	+.945 S	+.172	-.104	-.139	-.014



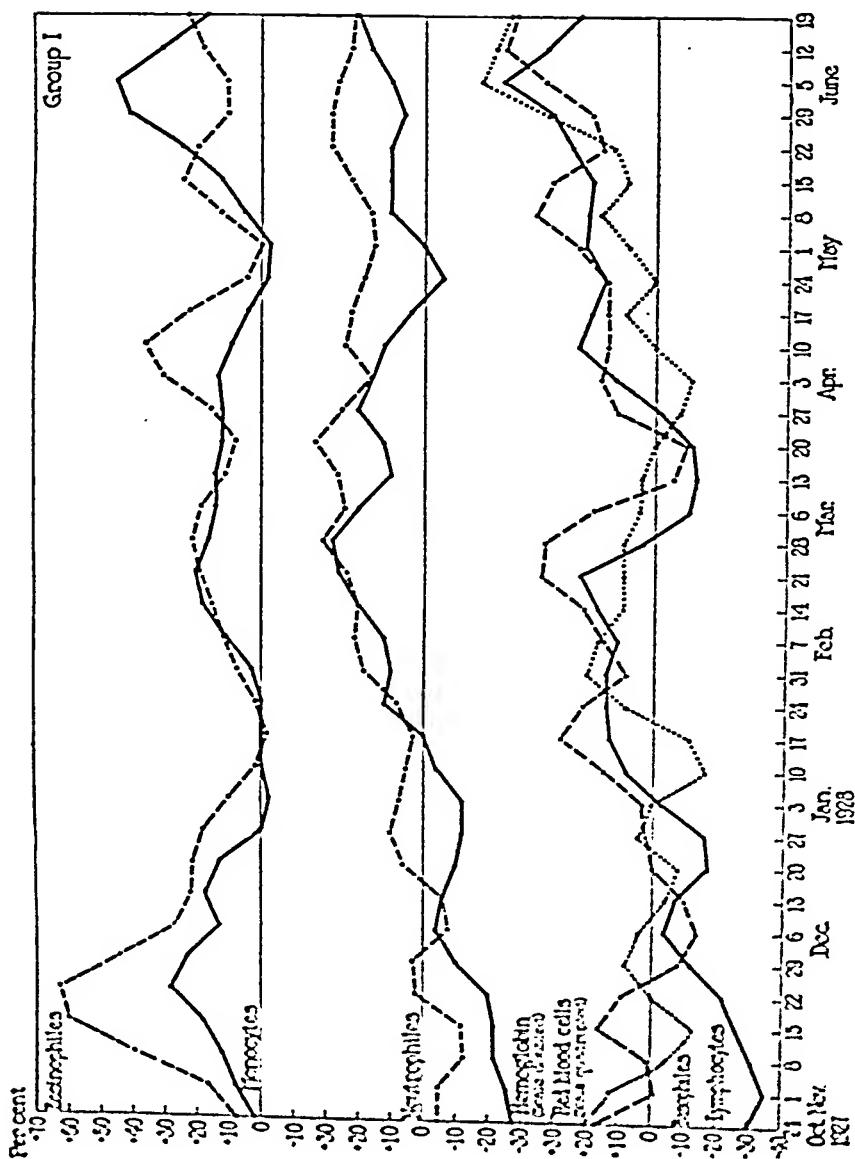
TEXT-FIG. 2

TEXT-FIG. 2. Mean values for consecutive determinations of blood constituents as percentage deviations from standard values. Group II

TEXT-FIG. 3. Mean values for consecutive determinations of blood constituents as percentage deviations from standard values. Group I



TEXT-FIG. 3



TEXT-FIG. 1. Mean values for consecutive determinations of blood constituents as percentage deviations from standard values. Group I.

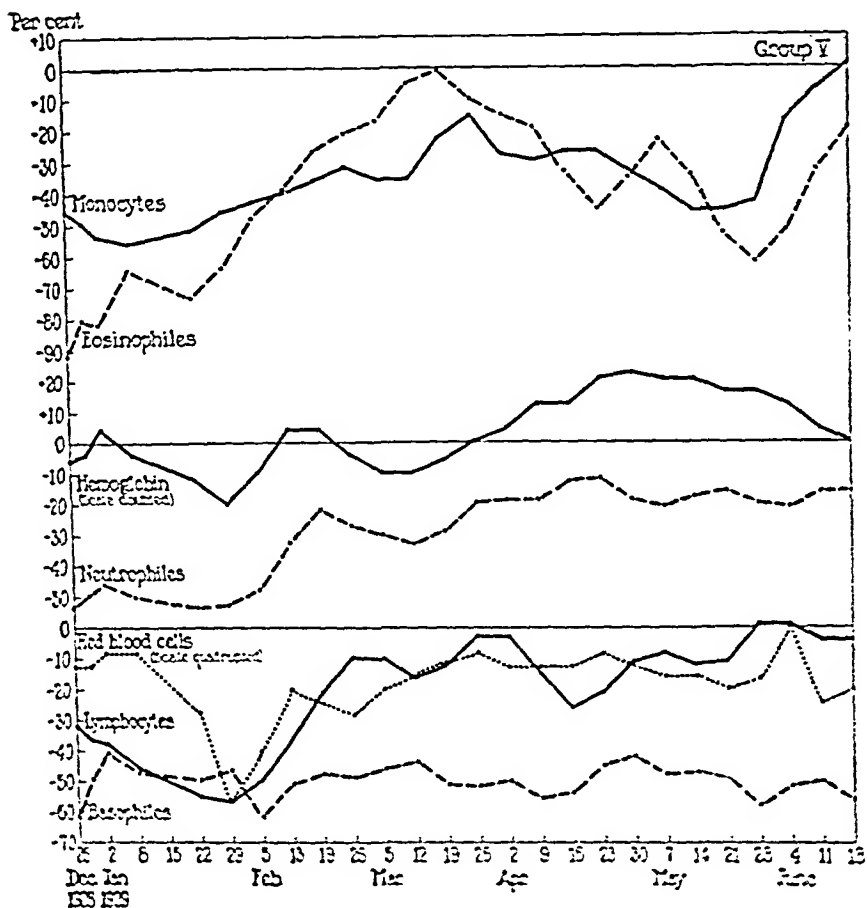
DISCUSSION

In this paper an attempt is made to evaluate comparatively small numerical variations of the blood cells which are ordinarily ascribed to technical error and assumed to be within the limits of "normal" variation. The particular object of the analysis was to ascertain whether any relationships in the movements of cell levels could be demonstrated. The data were obtained from four groups of ten and one group of five normal rabbits examined for prolonged periods of time; weekly mean values have been used as the basis of the study. Although larger groups and parallel observations would have been desirable, it is believed that the available material suffices for the present purpose.

It is conceivable that the group means of any two blood constituents may vary with time in a variety of ways. For example, the general numerical level of two classes of cells might increase or decrease over 7 or 8 months so that a coefficient of correlation upon their smoothed curves would be very high. Regardless of this general trend, however, there may be a number of abrupt oscillations of short duration in which the two levels fluctuate in the same or opposite direction, or bear no apparent relation to each other. This short period change we have called "fluctuation or oscillation" in contradiction to the term "trend" which is used to describe a progressive change over a period of months.

Table I contains the simple zero order coefficients of correlation which bring out the feature of cell relationship from the standpoint of trends in values over periods of months. All five groups are included although the eight observations of Group III at irregular intervals do not offer the same opportunity for comparison afforded by the other groups. Group II was examined regularly but only for 13 weeks, and Group IV suffered more heavily than the others from spontaneous disease. Bearing these points in mind, it will be noted that Groups I and V contain 16 and 14, and Groups II, III, and IV, 10, 8, and 2 significant coefficients respectively. The inclusion or exclusion of Groups II and III, however, makes no appreciable difference in the combined correlation coefficients as will be seen by comparing the figures in the last column of the table.

smoothed weekly means from so called standard values¹ for four of the five groups of rabbits; Group III is omitted because it was irregularly followed and contains only eight observations. No curve for the total white blood cells is given because this value does not represent an individual cell entity. The arrangement of



TEXT-FIG. 4. Mean values for consecutive determinations of blood constituents as percentage deviations from standard values. Group V.

charting monocytes with eosinophiles, neutrophiles with hemoglobin, and red blood cells with lymphocytes and basophiles, illustrates certain associations to which attention will later be directed.

¹ The standard values employed were obtained from 1110 counts on 176 normal male rabbits carried out in this laboratory (1).

neutrophiles, the basophiles, the eosinophiles, the lymphocytes, and the monocytes. Since there are seven variables under consideration, the relation between any two can be determined with the other five factors held constant.

In Table III, the combined zero order trend correlations for all five groups are given in the first column; the partial fifth order correlations upon these appear in the second column; the combined variate sixth difference correlations for all groups with the exception of the too short third group are given in the third column; and the partial fifth order correlations upon these last values comprise the fourth column. Since the combined correlation coefficients do not represent in every case an homogeneous material, too much emphasis should not be placed upon either these values or their derived partial coefficients. Partial correlations upon the individual groups, however, give approximately the same values as those based upon the combined values.

With the results of these analyses at hand (Tables I, II, and III), the relationships of the several types of cells may now be described. Among the 28 possible relationships, one finds that the white blood cells are highly associated with the neutrophiles and lymphocytes, both as regards general trend and oscillation; and that they are also related with the movements in level of the monocytes, the red blood cells, and the basophiles, in the order named. From the standpoint of cell number alone, such an arrangement of correlations would be expected as far as the white blood cells are concerned although the significant relation of the white blood cells with the red blood cells introduces the question of blood volume change.

The red blood cell levels varied from week to week and from month to month in all groups of animals. In these variations, they were accompanied most closely by the basophiles, the lymphocytes, the hemoglobin, and the monocytes in the order named; no significant homogeneous relation to the neutrophiles or the eosinophiles was found. The combined coefficients representing all groups were the same both for trend and for oscillations. Partial correlation affected very little the combined values of the simple or variate difference correlations, except that the neutrophiles and the eosinophiles showed a possibly significant negative relation, though of an irregular nature, and the relation with the lymphocytes was somewhat reduced. Even

Only a tentative value can be assigned to the combined correlation coefficients for all five groups (Table I) inasmuch as Group I had six coefficients significantly higher and Group IV, ten coefficients significantly lower than the combined values. What effect spontaneous disease conditions had upon the blood cell values of Group IV is hypothetical, so that this group cannot fairly be eliminated. The chronic basopenia in Group V with the lack of any trend whatsoever is responsible for the absence of conformity of these cells in the relationships of this animal group. In general, the various relationships were most similar in Groups I, II, and V, and coefficients averaged from this material would show only two or three significantly different values.

Table II represents an attempt to measure the relations between the cells of the various animal groups as regards short term fluctuations or oscillations by means of the variable sixth difference method. In this analysis, trend is practically eliminated. Since at least 10, and preferably more than 25 observations are necessary for dependable coefficients of correlation, Groups II and III were too short to be treated by this method; Group II is included in the table to show that certain spurious results can be obtained by using so short a group. Inclusion or exclusion of Group II in the combined coefficients, however, makes no significant difference in the results. The results of the analysis show that Group I had 4, Group IV, 6, and Group V, 5 coefficients significantly different from the combined values.

With time trend correlations such as those of the present study, two values may seemingly be related to each other in time, due to a common influence, although actually they may be unrelated. To eliminate this possibility, a third factor or factors should be found which are also similarly affected by this common influence. In this series, it has been assumed that for any simple time relationship between two variables, the remaining variables constitute to a varying degree such a third factor or factors. We have taken the combined correlation coefficients for both trend and short time fluctuations in each of the 21 simple relationships and computed the partial correlations of the highest order possible, that is, the fifth order. Partial correlations with total white blood cells as a separate entity would be inaccurate since this total value is a summation of the values for the

evidence while no apparent relation to the neutrophiles and hemoglobin was detected.

The lymphocytes were not significantly related to any of the other blood elements in regard to short term fluctuations, except perhaps to the basophiles and the red blood cells. However, there was a tendency toward a negative relationship with the neutrophiles and the eosinophiles but none whatever with the hemoglobin and the monocytes. With regard to trend, the lymphocytes were positively and significantly related to the red blood cells, the neutrophiles, and the basophiles, but not at all to the hemoglobin and the monocytes; there was a slight negative relation with the eosinophiles. The only significant difference in the intercellular relationships of the lymphocytes both as to trend and oscillation occurred with the neutrophiles.

The monocytes were found to be positively related both as to trend and oscillation to the red blood cells, the neutrophiles, and the eosinophiles; they were significantly inversely related to the hemoglobin and insignificantly inversely related to the basophiles. No relationship of the monocytes and lymphocytes with respect to either trend or oscillation was found.

The multiple correlation coefficients in Table IV furnish information on two important points, first, the total association of a blood cell type with other given blood cell types, and second, the total association due to unaccounted for factors. The value R^2 is a rough estimate of the total association of any one cell with other cells, the relationships being considered in percentage values. It will be seen, as was to be expected, that all the total white cell variations both as regards trend and fluctuation, can be attributed to variations in the component cell types. About 50 per cent of the variations in the red blood cells and hemoglobin, and from 21 to 47 per cent in the case of the eosinophiles, lymphocytes, and monocytes are explained by this relation to other cells; in the case of the neutrophiles and basophiles, depending upon the type of fluctuation considered, an irregular amount is similarly accounted for. It should be noted that with the basophiles and the eosinophiles, the total associations as shown by the variate difference method are twice as large as those shown by the simple correlations. Conversely, the values for neutrophiles and lymphocytes are one-third less by the variate difference method. The nature of the material

when the question of lag was considered, the neutrophiles and the eosinophiles did not seem to shift with the red blood cells. However, by neither method was the value for the neutrophiles homogeneous or consistent among the component groups. Perhaps one of the most striking points brought out by the analyses is the fact that the hemoglobin content did not always follow the values of the red blood cells.

The hemoglobin content varied in all groups. It was significantly and homogeneously related to the red blood cells both as to trend and fluctuation. In the short term fluctuations or oscillations, a significant negative relation to both monocytes and neutrophiles occurred which was independent of other intercellular relationships. In respect to trend, however, the mean percentage of hemoglobin was directly related to the neutrophiles and inversely related to the monocytes. It was never significantly related to the basophiles, the eosinophiles, or the lymphocytes.

The neutrophiles largely determined both the trends and the fluctuations of the total white blood cells. With regard to trend, the neutrophiles were directly and significantly related to the mean values for the hemoglobin, the lymphocytes, and the monocytes in the order named, but were seemingly unrelated to the eosinophiles and basophiles, and inversely related to the red blood cells when other blood factors were held constant. As to fluctuation or oscillation, the neutrophiles were significantly but not always related to the red blood cells, and bore insignificant negative relations to the hemoglobin, the basophiles, and the lymphocytes; there was no relation whatsoever to the eosinophiles. Although the relation with the monocytes was homogeneous among the component groups and significant for the zero order values, this significance disappeared upon partial correlation.

The basophiles were fairly homogeneously, significantly, and positively related both as to trend and oscillation with the red blood cells, the eosinophiles, and the lymphocytes. Opposing this were small negative relations with the hemoglobin, the neutrophiles, and the monocytes.

The eosinophiles were found to be directly and most closely related, first, to the basophiles and second, to the monocytes. A small negative relationship to the red blood cells and lymphocytes was in

while the neutrophiles and especially the hemoglobin were maintained at comparatively constant levels.

The various classes of cells and the hemoglobin in the several animal groups seem to bear a general similarity of relationship to the standard mean values, that is, all seven elements were either above or below the standard value at the same time. The most frequent exceptions occurred in the case of the neutrophiles, the red blood cells, and especially, of the hemoglobin. This fact would seem to indicate that the hemoglobin, the neutrophiles, and the red blood cells were either less disturbed by common influences affecting shifts in the other cells, or that a compensatory mechanism exists for these blood constituents which operated in such a manner that less marked deviation from the level of these standard values occurred. Successive examinations of the fluid volume of the blood might throw light on these points.

At the present time it seems undesirable to attempt to estimate the significance of these results as they represent only a 2 year period in one species in a single laboratory. It is evident that before any interpretation of the results can be attempted with any general conclusions, supplementary evidence of a similar sort should be obtained.

SUMMARY

1. Statistical analyses have been made of the weekly variations in the blood counts of groups of normal rabbits to find whether there exists any relationship between the numerical changes occurring in the various cell types.

Consecutive blood counts and differential white cell determinations on five groups of normal male rabbits comprising 45 animals in all were made at weekly intervals from October, 1927, to June, 1929, the number of observations on each group varying from eight to thirty-five.

2. The following relationships between the varying group means were found to be consistent and significant:—The number of the red blood cells varied with the amount of hemoglobin per cubic millimeter and with the number of lymphocytes. There was an inverse relationship between the amount of hemoglobin and the number of monocytes. The neutrophiles varied in number with the monocytes; the basophiles with the eosinophiles; and the eosinophiles with the monocytes.

and the methods of analysis employed justify only the most general interpretations, but it may be stated that from 40 to 65 per cent of the variations in all the blood constituents here considered seem unaccounted for on the basis of common associations or as caused by the operation of a common factor.

The curves of the text-figures which represent the percentage deviations of the smoothed weekly means from standard values have been arranged so as to show the largest number of significant cell relationships. It should be pointed out, however, that several high associations are not well shown by this arrangement, as for example, the red blood cells and the hemoglobin, the neutrophiles and the monocytes, the monocytes and the hemoglobin, and the basophiles and the eosinophiles. In Group I (Text-fig. 1) the eosinophile and monocyte curves are above the standard values for the entire period of observation while in Groups IV and V of the following year (Text-figs. 3 and 4) they are consistently below these values. In Group II (Text-fig. 2) which paralleled Group I in its latter half, the eosinophiles and the monocytes were also below the standard values but intermediate between the findings in Group I and Groups IV and V. But irrespective of these wide variations in general level during the 2 years' observations, the curves illustrate in a striking manner the close relationships between monocytes and eosinophiles, not only in respect to the general trend of mean values, but also as regards the shorter fluctuations, a fact which was brought out by the correlation coefficients already discussed. The same conclusions may be drawn from the curves illustrating the red blood cells, the lymphocytes, and the basophiles on the one hand, and those representing the neutrophiles and the hemoglobin on the other. During the late winter and spring and early summer months of 1929, there was a chronic basopenia in both animal groups examined over this period, and this shift in level was perhaps the most striking one observed. In contrast to these changes in cell levels, the hemoglobin and the neutrophiles vacillated about the standard level during the entire period. From the standpoint of general trends or shifts in the numbers of cells during the two year period from October, 1927, to June, 1929, the eosinophiles and the monocytes and the lymphocytes, the basophiles, and the red blood cells of successive animal groups sought progressively lower levels

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Other associations not always similar but of high significance as far as the combined values were concerned, were the relations of the red blood cells with the basophiles and the monocytes. The relations of the neutrophiles with the red blood cells and the hemoglobin were very irregular.

3. Significant association of the white blood cells with variations in the red blood cells and the hemoglobin content were observed. The numerical variations in the group means of the total white cells were associated with similar variations in the group means of the neutrophiles, the lymphocytes, the monocytes, the basophiles, and the eosinophiles almost to the degree of their numerical occurrence in the peripheral blood.

4. With the exception of the total white cells, approximately only half the variations in the group levels of the various cells and of the hemoglobin content can be accounted for on the basis of simultaneous associations with each other.

5. The red blood cells, the lymphocytes, and the basophiles as one group, the eosinophiles and the monocytes as another group, and the hemoglobin content and the neutrophiles as a third group, described a definite shift from a high to a low numerical value during the 2 year observation period. From the standpoint of the magnitude of the shift, the basophiles, the eosinophiles, the monocytes, the lymphocytes, and the red blood cells participated in the order mentioned. The neutrophiles were only slightly affected and the hemoglobin content relatively not at all.

6. No significant relationship was ever found, even in the component groups, between the weekly mean values of the following: the hemoglobin with the basophiles, the eosinophiles, or the lymphocytes; the neutrophiles with the basophiles or the eosinophiles; and the lymphocytes with the eosinophiles or the monocytes.

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5. Pearce, L., and Casey, A. E., *J. Exp. Med.*, 1930, 52, 167.

EXPERIMENTAL

The two transmissible strains described here originate from two spontaneous cases observed in a stock of albino mice designated A. This stock was purchased from a dealer and is being bred in our laboratory for the study of leucemia. Two other stocks of mice, called R and S, have been secured from different sources and are being bred under similar conditions in order to procure spontaneous cases of the disease. Failure to obtain a strain of mice in which leucemia is known to occur necessitated this laborious undertaking. Each mouse is examined for enlargement of lymph nodes and spleen at weekly intervals. The superficial lymph nodes and the spleen are easily palpable when

TABLE I

Incidence of Spontaneous Leucosis in the Stocks of Mice Used in Transmission Experiments

	Number of mice* examined		Number of cases of lymphoid leucosis	
	Living	Dead	Leucemic	Aleucemic
Stock A.....	350	220	2	2
Stock R.....	600	420	2	0
Stock S.....	250	470	1	4

* Above the age of 8 months.

they are distinctly enlarged. Blood smears are taken from all mice that have enlarged lymph nodes and from the inoculated mice at intervals of 1 or 2 weeks.

Spontaneous Leucosis in the Stocks of Mice Used for Transmission Experiments.*—Leucosis occurs in all three of our stocks, but it is very infrequent.† The incidence of spontaneous leucosis in our laboratory has not as yet been exactly determined; the accompanying approximate figures may be of value in interpreting the transmission experiments (Table I).

Four cases of leucemia were discovered during life, one in Stock S,

* Leucosis is used as a collective term for leucemia, lymphoma and related conditions.

† At the time a preliminary report (7) of these investigations was published, leucemia had been observed only in Stock A.

STUDIES ON TRANSMISSIBLE LYMPHOID LEUCEMIA OF MICE*

BY J. FURTH, M.D., AND M. STRUMIA, M.D.

(From The Henry Phipps Institute and the Department of Pathology, University of Pennsylvania, Philadelphia)

PLATES 23 AND 24

(Received for publication, February 5, 1931)

One may expect studies on the transmissibility of leucemia to show whether it is an infectious, hyperplastic or neoplastic process and to determine the much debated etiological relation between leucemic and aleucemic lymphadenosis and lymphoma.

Frequent attempts to transmit leucemia in mammals, reviewed by Opie (1), have been made without success, until recently Snijders (2) transmitted lymphoid leucemia of guinea pigs. By intraperitoneal inoculations of blood or emulsions of organs, Snijders reproduced leucemia in 58 successive generations. The majority of the guinea pigs successfully inoculated developed leucemia, the rest, aleucemic lymphadenosis with or without tumor formation at the site of inoculation. He observed that transmissions were unsuccessful with filtrates free from cells. Snijders' investigations have been fully confirmed by Tio Tjwan Gie (3).

Richter and MacDowell (4) transmitted leucemia in a highly inbred strain of grey mice, of which almost every animal that lived longer than about 8 months developed leucemia spontaneously. Young mice of this strain, when inoculated intraperitoneally or subcutaneously with an emulsion of blood or lymph nodes of leucemic mice, developed leucemia within a few days; mice unrelated to them were found to resist similar inoculations. By crossing susceptible mice with mice resistant to transmissible leucemia MacDowell and Richter (5) reached the conclusion that susceptibility to transmission is inherited as a Mendelian dominant character.

Korteweg (6) inoculated mice intraperitoneally with an emulsion of a spontaneous lymphosarcoma of the mediastinum of a mouse. He observed the development of a lymphoid tumor at the site of inoculation in about 25 per cent of the inoculated animals and most of these showed a terminal leucemic blood picture. Attempts at transmission by cell-free material were unsuccessful.

* This investigation has been supported by a Fund for the Study of Leucemia and Related Diseases.

TRANSMISSIBLE LYMPHOID LEUCEMIA OF MICE

TABLE II

Transmissions from Spontaneous Leucemia A 8

Transfer from mouse No.	Passage	Stock A, intravenous			Stock A, intraperitoneal			Stock A, subcutaneous			Stock R, intravenous			Stock S, intravenous		
		No. injected	Leucosis		No. injected	Leucosis		No. injected	Leucosis		No. injected	Leucosis		No. injected	Leucosis	
			Leucemic	Aleucemic		Leucemic	Aleucemic		Leucemic	Aleucemic		Leucemic	Aleucemic		Leucemic	Aleucemic
A 8	Orig.	22	2	0	15	0	0	15	0	0						
A 246	I	32	5	2**	18	1	1									
A 209	I	12	1	0	15	0	1									
A 40	II	18	3	0	9	0	0									
A 50	II	14	1	0	5	0	0									
A 96*	II	9	0	0							8	1	0			
A 931*	II	18	2	1				6	0	0						
A 181	III	14	3	0	16	0	0									
A 179	III	19	2	0	2	0	1									
R 135	III															
A 805*	III	25	1	1**							26	6	2			
A 804	III	6	0	0				23	0	0						
A 1101	IV	8	1	0												
A 1239	IV	9	2	0										12	0	0
A 1240	IV	12	0	0							7	1	1			
A 220	IV	25	0	0	10	0	0							15	0	0
R 771	IV										13	0	0			
R 774	IV										11	0	0			
R 1742	IV	2	2	0												
A 837	IV	9	0	0				8	0	0						
A 1383	V	25	1	1												
A 1297	V	24	6	1												
A 1398	V	17	1	1												
A 1519	VI	13	2	0												
A 1396	VI	3	3	0												
A 1534*	VI	29	0	0												
A 1707	VII	6	1	0										3	2	0
A 1633	VII	13	0	0										16	0	0
A 1738	VIII	5	0	0										9	0	0
Total...		389	39	7	90	1	3	52	0	0	65	8	3	61	2	0

* These donors were aleucemic; all the others leucemic.

** One of these may be spontaneous for it developed several months after inoculation.

one in Stock R and two in Stock A. Only the last two cases were transmitted successfully.

Origin of the Transmissible Strain. Methods of Transfer

The donor of the first transmissible strain (A 8) was about 15 months old when a uniform enlargement of superficial lymph nodes so that each measured about 0.6 to 1 cm. across was noted. The microscopic picture of the lymph nodes showed the extensive lymphoid hyperplasia (Fig. 1) characteristic of lymphoid leucemia. The spleen was very much enlarged and extensive lymphoid infiltrations were found in the liver (Fig. 2) and in the kidney (Fig. 3). The white blood cells numbered 315,000; 68 per cent were lymphocytes and of these about 30 per cent showed signs of immaturity. The transmissions were made by injecting blood, to which heparin had been added to prevent clotting, or a fairly turbid suspension of lymph node tissue into the tail vein of normal mice. The material from lymph nodes was obtained by cutting them up with small scissors in the presence of Locke's solution and filtering through a small piece of cotton. In several parallel series the susceptibility of our three stocks to inoculations by the subcutaneous intraperitoneal and intravenous routes were compared.

The second transmissible strain was derived from a spontaneous case (A 984), which resembled the first case very closely. It was transmitted in a similar manner. The passages of the first strain are collected in Table II, those of the second strain in Table III.

The success of the inoculations varied with the individual passages. *E.g.*, both mice inoculated intravenously with leucemic blood of R 1742 and all three mice inoculated in a similar manner with leucemic blood of A 1396 developed leucemia. The blood in these cases was obtained from the heart of the animals and was injected in amounts of 0.05 to 0.15 cc. On the other hand, several other attempted transfers from other leucemic mice made in a similar manner were entirely negative. In one instance 4 of 10 mice inoculated intravenously with an emulsion of lymph nodes of Mouse R 1684 developed leucemia whereas a second similar transfer attempted 9 days later from the same donor to 13 mice was entirely unsuccessful.

A comparison of the intravenous transmissions with blood and with lymph nodes in Stocks A and R gives the following figures:

	No. injected	Leucemia		Successful inoculations
		Leucemic	Albino	
Inoculated with lymph nodes	463	53	14	13.5
Inoculated with blood	133	16	6	12.5

lations in the 8 mice not known to be closely related to a spontaneous case of leucemia were successful.

Notably unsuccessful were the attempts at transmission during the extremely hot summer of 1930, resulting in a loss of the transmissible strains described in this report. The negative inoculations made at that time during our absence, have been included in Tables II and III; they are responsible in part for the low mean percentage of successful inoculations.

The figures presented in Tables II and III show that Stocks A and R are equally susceptible to transmissible leucemia. It is noteworthy that Stock R was secured as a well observed normal stock in which the incidence of cancer was found to be low and leucemia was not known to occur. Stock S appears somewhat less susceptible although the data presented in Tables II and III are not sufficient for us to estimate its relative susceptibility. When transfers to Stock A were effective, mice of Stock S reacted well to simultaneous inoculations. Most of the transfers to Stock S were attempted during the summer of 1930 when some undetermined factor interfered with the success of inoculations. For this reason in the analysis that follows Stock S will not be considered.

Comparison of Intravenous, Intraperitoneal and Subcutaneous Inoculations. *Etiological Relationship of Leucemic and Aleucemic Leucosis.*—It is evident from Tables II and III that leucosis can be best transmitted by intravenous injections. The majority of the mice successfully inoculated by this route developed leucemia, namely 69 of the 726 mice inoculated or 9.5 per cent, and a smaller number, 21 (2.8 per cent), developed aleucemic lymphadenosis. Much less successful was attempted transmission by the intraperitoneal route; only 1 of 124 mice injected developed leucemia and in 3 there was a lymphoid tumor unaccompanied by a systemic enlargement of the lymph nodes. In 2 of these the tumor was situated at the site of inoculation (A 931 and A 1156) and in one (A 707) in the mediastinum. The latter case may have been spontaneous. Subcutaneous inoculations into 62 mice were entirely negative.

The figures presented in Tables II and III give sufficient evidence for the possibility of the development of aleucemic lymphadenosis by inoculations with organs of leucemic mice. The reverse possibility, namely the production of leucemia by intravenous inoculation of or-

The factors governing the outcome of a transmission are not fully known. Some of them will be discussed later; others, such as the amount of inoculum necessary to produce leucemia, the influence of the medium used in its preparation, the physical condition of the recipient etc. have not as yet been sufficiently investigated. That hered-

TABLE III
Transmissions from Spontaneous Leucemia A 984

Transfer from mouse No.	Passage	Stock A, intravenous			Stock A and R, intraperitoneal			Stock R, intravenous			Stock S, intravenous		
		No. injected	Leucosis		No. injected	Leucosis		No. injected	Leucosis		No. injected	Leucosis	
			Leucemic	Alcemic		Leucemic	Alcemic		Leucemic	Alcemic		Leucemic	Alcemic
A 984	Orig.	14	1	0	(A) 10	0	0	18	1	1			
R 639	I							16*	1	1			
A 682	I	16	0	0	(R) 9	0	0						
					(A) 6	0	0	12	0	0			
R 1694	II							23	4	0			
R 1657	III							16	1	0			
R 1659	III							7	0	3	9	0	0
R 1663	III	6	1	0				23	4	3			
R 1660	III	16	6	0				9	1	0	7	0	0
A 1497	IV	15	2	0							20	2	1
R 1278	IV	17	0	2							13	1	0
A 1364	IV	21	0	0									
S 2457	V	10	0	0									
A 1612	V	6	0	1									
S 1678	V										9	0	0
S 2362	V	27	10	0							18	0	0
Total....		148	10	3	25	0	0	124	12	8	76	3	1

* 8 mice of Strain R were inoculated subcutaneously with this material without success.

itary susceptibility is an important factor has been demonstrated by MacDowell and Richter. It is noteworthy in this connection that in a passage (from R 1660) 8 of the 16 mice inoculated were grandchildren of A 8, an animal with spontaneous leucemia. Four of these 8 mice developed leucemia after inoculation, whereas only 2 of the inocu-

TRANSMISSIBLE LYMPHOID LEUCEMIA OF MICE

There is a systemic enlargement of the lymph nodes, the size of which frequently exceeds 1 cm. in diameter. The lymph follicles of the intestinal tract are very prominent and the size of the mesenteric lymph nodes usually exceeds those of the superficial lymph nodes. The retroperitoneal lymph nodes form frequently a confluent mass embedding the adrenal, parts of the kidneys and adjacent structures. Similarly the lymphoid mass extending from the mediastinal lymph nodes not infrequently covers most of the heart. The spleen measures about 3 to 4 cm. in its longest diameter. The microscopic picture of the lymph nodes and spleen is illustrated in Figs. 5 and 6. The lymphoid infiltration of various organs seen in spontaneous leucemia (Figs. 2 and 3) are equally characteristic for leucemia produced by transmission (Fig. 7).

The microscopic picture of the organs involved has the character of a highly invasive neoplastic growth composed of medium and large cells resembling lymphocytes, many of them in mitotic division.

The anatomical changes in aleucemic lymphadenosis were essentially the same, except that the circulating blood was not invaded.

In the cases with tumor formation at the site of inoculation (peritoneal cavity) several lymph nodes were considerably enlarged but the liver was not infiltrated. Fig. 8 is a view of the tumor A 931 described above.

Relation of Age to the Result of Inoculation.—Table IV records the results of the inoculations with transmissible leucosis in the mice grouped according to age.

TABLE IV

Relation of Age to the Results of Inoculation with Transmissible Leucosis

Age in mos.	Below 3	3 to 5	5 to 7	7 to 9	9 to 11	11 to 13	Over 13
No. of mice inoculated.....	612	218	124	70	86	32	20
No. of successful inoculations..	48 (9)*	13 (1)	10 (3)	0	17 (5)	3 (2)	1
Percentage of successful inoculations.....	7.8	6	8	—	19.8	9	5

* The figures in parentheses indicate the number of cases of leucosis with aleucemic blood picture.

It would seem from Table IV that young as well as old mice may succumb to leucosis if leucotic material is introduced into the circulation. The age of mice with spontaneous leucemia observed in these stocks was from 12½ to 18 months; it appears from this table that mice

gans from aleucemic cases, has been tested by inoculations from four cases (A 96, A 931, A 805 and A 1534, Table II), only twice with success.

One of these transfers was made from the above mentioned Case A 931, a mouse with a white blood count of 15,000 to 32,000 during a 2 weeks' period of observation. Two of 18 mice inoculated intravenously with an emulsion of the lymphomatous tumor of A 931 developed leucemia and one (A 805) aleucemic lymphadenosis. A 805 was under observation for 46 days following the discovery of enlarged lymph nodes measuring 0.6 to 1 cm. across. The white blood count varied from 12,500 to 27,000. The postmortem appearances were those of aleucemic lymphadenosis with pronounced lymphoid infiltration in various organs. Following intravenous inoculation of 25 mice with an emulsion of lymph nodes of this aleucemic mouse one of the inoculated mice developed leucemia.

These three cases of leucemia developed 14 to 19 days following intravenous inoculation of lymphoid tissues derived from aleucemic cases. The incubation period and the youth of these mice (4 to 6 months) almost certainly excludes the possibility that they were spontaneous.

These observations are in harmony with those described by the investigators mentioned above, indicating that leucemic and aleucemic lymphadenosis may occur with or without tumor formation and that they are varieties of the same condition. One factor that appears important in determining the type of leucosis is whether the leucotic cells have a free entrance into the circulation, for intravenous dissemination seems to favor leucemia. Another determinant would seem, according to the studies of Richter and MacDowell, to be the character of the cells of the donor (8). These investigators observed that the lesions produced by inoculation of lymph nodes deriving from several spontaneous cases show considerable differences depending solely on the cells of the donor.

Anatomical Changes in Transmissible Leucemia of Mice

It is not within the scope of this work to describe in detail the gross and microscopic appearance of the organs in leucemia and lymphoma produced by transmission. Leucemia as produced by intravenous inoculations resembles spontaneous leucemia very closely.

TRANSMISSIBLE LYMPHOID LEUCEMIA OF MICE

in the leucemic blood. A few days after the appearance in the blood of lymphoblasts, the total number of leucocytes begins to increase (Tables VI, VII) with a continuous relative as well as absolute rise of the number of lymphoblasts. The peak, relatively speaking, of the lymphoblasts, is reached before the total leucocyte count is at its

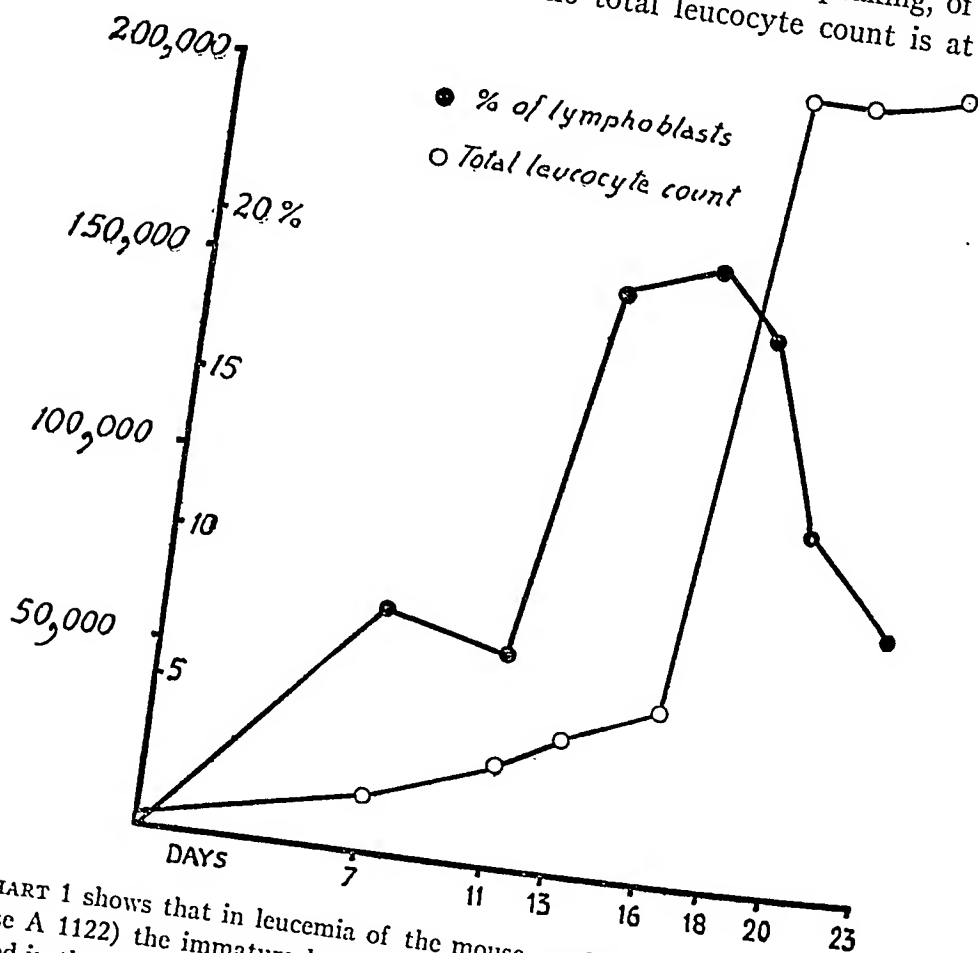


CHART 1 shows that in leucemia of the mouse produced by transmission (in Mouse A 1122) the immature lymphocytic cells (lymphoblasts) are greatly increased in the circulating blood before the total number of leucocytes reaches its maximum.

maximum, as is illustrated by the accompanying graph (Chart 1). The percentage of undifferentiated cells at the peak varied from 12 to 35 per cent.

The percentage of lymphocytes closely paralleled the total leucocyte increase. Polychromatophilia increased greatly with the development

are most susceptible to inoculations during the period immediately preceding, when they are from 9 to 11 months old. However the number of mice in each group is too small to form a basis for a definite opinion as to the relation between age and susceptibility to leucemia.

The Blood Picture in Transmissible Leucosis

The average count for normal mice of the breeds used was neutrophilic polymorphonuclears, 42 per cent, immature granulocytes (metamyelocytes, ring forms), 3.6 per cent, lymphocytes, 52 per cent, lymphoblasts, 0.4 per cent, "pro-lymphocytes,"* 1 per cent, monocytes, 1 per cent. Polychromatophilia is common in these apparently healthy stocks. Only one typical eosinophile was seen in a very large number of slides examined. The percentage of lymphocytes varied within very broad limits (from about 25 to 72 per cent) and under many conditions, such as age and external temperature. No less variable was the total leucocyte count, the figures ranging between 2,000 and 16,000, with an average of about 8,000. However the figures below about 5,000 were found in a single day when the temperature was unusually high. If these counts are disregarded the average is about 11,000.

The changes in the blood picture occur rather suddenly, the first definite leucemic blood change being a sharp relative increase in the number of immature lymphocytes or lymphoblasts preceding the leucocytosis (Tables VI and VII). These are rather large cells (18 to 24 μ) with dark blue cytoplasm, with no granules or a few fine azurophilic granules usually in the perinuclear zone, a large round or slightly oval nucleus with a very fine chromatin network and 2 to 5 nucleoli poorly differentiated in smears stained with Romanowsky stains. These lymphoblasts appear to differ somewhat from those occasionally observed in the normal circulating blood because of their larger size and the more pronounced basophilia of the cytoplasm. Biologically a distinct difference is shown by the capacity of the leucemic lymphocytes to produce leucemia. (Cf. Korteweg.) The "pro-lymphocytes" normally present in the blood (1 per cent) diminished at least relatively

* Very small lymphoid cells with narrow rim of intensely basophilic cytoplasm without granulation and with a very compact structureless nucleus (Ferrata).

of the leucemic picture, as did the number of "shadow" cells (disintegrated nuclear bodies).

It is of great significance that almost all of the lymphocytes found in leucemic blood smears are more or less immature, although not immature enough to justify their classification as lymphoblasts. They are larger than normal lymphocytes, the cytoplasm is deeper in color and the nucleus has a finer structure with greater variations than occur in normal cells. The number of cells with azurophilic granules in the cytoplasm is also decidedly less.

In the cases of aleucemic lymphadenosis observed after the inoculation of leucemic blood the percentage of lymphoblasts was not so high as in the pre-leucemic stage of transmitted leucemia, as is shown in Table V.

Observations on the Pathogenesis of Leucemia

The first evidence of a successful inoculation was a uniform enlargement of all superficial lymph nodes observed from 7 to 25 days following the intravenous inoculation. The blood counts taken at this stage of the disease were normal; the differential counts showed a percentage of lymphocytes within the normal range, but the percentage of lymphocytes with signs of immaturity was as a rule above normal. Within a few days the number of the circulating leucocytes rose considerably and the aleucemic phase was soon followed by typical leucemia (Table VI). In a few instances the blood count was already leucemic when the enlargement of the lymph nodes was discovered (Table VI); this observation however was of little significance because the physical examinations were made only at 3 to 4 day intervals and the aleucemic stage could easily have been missed.

Thus it appears that when leucemic cells are injected into a vein of normal mice they leave the circulation and multiply in certain tissues favorable for their growth. The occasional high percentage of immature lymphocytes in the circulating blood during the aleucemic phase suggests that immature cells enter the circulation steadily but presumably leave it to "colonize" in some other location. This mechanism would seem adequate to explain the uniform enlargement of all lymphoid tissues.

The history of a small group of mice closely followed from the day

TABLE V
Blood Counts in Aleuemic Lymphadenosis

No. of mouse	Blood counts at time of the recovery of enlarged lymph nodes				Subsequent blood counts										No. of days* when killed (k.) or died (d.)	
	No. of days*	White blood count	Lymphocytes	Lymphoblasts	No. of days*	White blood count	Lymphocytes	Lymphoblasts	No. of days*	White blood count	Lymphocytes	Lymphoblasts				
A 1623	8	5,000	30	0	13	17,000	60	1	20	15,000	28	0	—	—	—	d. 22
R 673	27	20,500	65	4	—	—	—	—	—	—	—	—	—	—	—	d. 31
R 633	22	25,500	—	—	27	43,000	47	5	30	33,000	—	—	—	—	—	d. 32
R 1765	30	7,200	59	1	37	23,000	66	2	—	—	—	—	—	—	—	d. 44
R 1769	8	49,500	57	0	11	63,500	46	0	—	—	—	—	—	—	—	d. 16
R 1415	13	32,500	61	1	21	11,000	64	3	27	10,000	52	2	—	—	—	d. 32
R 1801	23	17,500	—	—	—	—	—	—	—	—	—	—	—	—	—	d. 30
A 805	20	15,000	—	—	25	14,500	42	0	49	12,500	—	—	—	27,000	50	k. 62
A 96	37	17,400	40	0	44	17,400	31	1	—	—	—	—	—	—	—	k. 44
A 911	41	13,000	58	1	—	—	—	—	—	—	—	—	—	—	—	d. 213
A 1125	22	15,000	58	1	28	23,500	62	0	33	30,000	64	1	35	37,500	51	0 d. 39
A 871	5	9,700	24	1	18	10,500	45	1	32	24,500	28	2	39	32,000	64	d. 116
A 1534	24	9,000	49	3	30	48,000	58	6	37	56,000	—	—	—	—	—	k. 37
A 931	36	15,000	—	—	43	16,000	60	8	50	32,000	71	5	—	—	—	k. 50
R 773	13	16,000	70	2	17	46,000	66	12	—	—	—	—	—	—	—	d. 23
R 784	27	23,500	59	3	35	46,000	50	2	42	45,000	63	3	49	56,500	49	d. 54
R 1059	21	18,500	28	0	28	24,500	33	0	—	—	—	—	—	—	—	d. 35

* After inoculation.

TRANSMISSIBLE LYMPHOID LEUCEMIA OF MICE

TABLE VII
The Development of Leucemia after Intravenous Inoculation

TABLE VII

The Development of Leucemia after Intravenous Inoculation

No. of mice.....		Inoculated mice					Control mice (uninjected)				
Before inoculation		A 1122		A 1297		A 1002	A 1344		A 1115		
7 days after inoculation	White blood count	2,600		2,200		3,800 41 0 Normal	1,500		3,600 46 0 Normal		
	Per cent of lymphocytes	32		32			40				
	Size of lymph nodes	0 Normal		0 Normal			0 Normal				
11 days after inoculation	White blood count	15,000		18,500		6,000 72 0 Normal	9,000		14,500 39 0 Normal		
	Per cent of lymphocytes	47		42			34				
	Size of lymph nodes	8 Very slightly enlarged		0 Normal			0 Normal				
13 days after inoculation	White blood count	27,600		27,500		7,800 70 0 Normal	12,000		20,500 41 0 Normal		
	Per cent of lymphocytes	53		32			46				
	Size of lymph nodes	7 3 to 4 mm.		2 Very slightly enlarged			0 Normal				
	White blood count	36,000		28,000		10,000 59 1 Normal	14,000		16,000 60 1 Normal		
	Per cent of lymphocytes	52		40			50				
	Size of lymph nodes	19 4 to 5 mm.**		1 2 to 3 mm.			0 Normal				

TABLE VI
Blood Counts in Leucemia Produced by Transmission

No. of mice	At time of the discovery of enlarged lymph nodes					Subsequent blood counts										No. of days* when killed (K.) or died (d.)	
	Size of lymph nodes	No. of days*	White blood count	Lymphocytes per cent	Lymphoblasts per cent	No. of days*	White blood count	Lymphocytes per cent	Lymphoblasts per cent	No. of days*	White blood count	Lymphocytes per cent	Lymphoblasts per cent				
A 1193	0.3-0.4	14	11,800	61	1	18	16,000	72	4	22	177,000	58	23	26	105,000	—	d. 30
R 1503	0.2	12	13,400	40	0	16	29,000	54	7	20	35,500	61	6	25	101,000	72	d. 27
A 1519	0.3	15	5,500	27	0	18	106,000	44	13	20	355,000	62	25	22	419,000	70	k. 22
A 813	0.5-1.0	16	19,200	31	2	20	36,000	58	3	25	135,000	50	24	28	295,000	47	d. 59
A 1101	0.3-0.4	18	11,500	45	3	20	32,500	39	23	24	174,000	67	16	28	222,000	69	19
A 122	0.3-0.5	16	8,000	75	4	23	116,000	56	24	35	325,000	55	27	32	407,000	88	5
R 619	0.2-0.4	22	13,200	68	12	27	32,000	78	12	30	125,000	76	14	42	187,000	—	d. 66
A 1051	0.5	22	10,000	41	4	26	60,000	37	17	32	153,000	52	17	—	—	—	d. 42
R 1661	0.2	14	11,000	66	4	22	35,500	61	3	29	130,000	60	17	41	284,000	55	d. 39
R 1775	0.2	16	11,500	71	5	21	20,000	71	6	24	25,000	63	16	28	66,000	59	k. 41
R 1502	0.2	12	5,800	30	2	16	46,000	66	2	25	103,000	42	11	35	212,000	74	d. 39
A 1210	0.2	17	22,500	41	14	20	48,000	60	19	27	226,000	70	20	29	516,000	68	d. 27
A 50	0.3	23	59,000	58	2	24	108,000	46	8	30	259,000	74	16	—	—	—	k. 29
A 926	0.3	16	44,000	56	21	23	174,000	80	16	28	258,000	75	18	43	252,000	72	d. 40
R 1735	0.4	15	32,000	58	4	23	196,000	73	17	27	220,000	75	15	—	—	—	d. 50
R 760	0.3-0.4	15	83,000	71	9	22	215,000	77	12	31	216,000	81	14	—	—	—	d. 28
R 771	0.4-0.5	15	285,000	68	13	23	280,000	73	13	24	485,000	—	—	—	—	—	d. 34
																	k. 24

* After inoculation.

of inoculation is fully given in Table VII. The leucocyte count of 3 control mice of the same age and breed, kept side by side with the inoculated mice, varied between 3,600 to 16,000, the percentage of lymphocytes from 41 to 77 per cent, the percentage of immature lymphocytes from 0 to 1 per cent. The first evidence of a successful inoculation was a very slight enlargement of all superficial lymph nodes observed 7 days after inoculation in one case and 11 days in another. At the same time there was a distinct increase in the percentage of immature lymphocytes (8 per cent in one and 2 per cent in the other). The absolute number of white blood cells as well as the percentage of mature lymphocytes appeared to be within the normal range.

SUMMARY AND CONCLUSIONS

Lymphoid leucemia of the mouse is readily transmitted by intravenous inoculations. The majority of the mice inoculated successfully develop leucemic, a smaller number of them, aleucemic lymphadenosis. The data presented favor the view that leucemic and aleucemic lymphadenosis are essentially the same condition.

Leucemia produced by transmission is preceded by an aleucemic stage, in which the lymph nodes and the spleen are uniformly enlarged, and the white blood count and the percentage of lymphocytes are within the normal range but immature lymphocytes are numerous in the circulating blood.

Young as well as old mice may develop leucemia if leucotic material enters their circulation.

Studies of transmissible leucemia favor the view that leucemia of mammals is a neoplastic disease. The basic problem of leucemia would seem to be determination of the factors that bring about a malignant transformation of lymphoid cells.

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16 days after inoculation	White blood count Per cent of lymphocytes Per cent of lymphoblasts Size of lymph nodes	46,000 62 20 4 to 5 mm.	24,000 58 1 3 mm.**	10,000 68 0 Normal	16,000 48 0 Normal	16,000 77 0 Normal
18 days after inoculation	White blood count Per cent of lymphocytes Per cent of lymphoblasts Size of lymph nodes	205,000 74 18 4 to 5 mm.	42,000 40 5 3 to 6 mm.	8,000 70 0 Normal	6,500 49 0 Normal	14,000 51 0 Normal
20 days after inoculation	White blood count Per cent of lymphocytes Per cent of lymphoblasts Size of lymph nodes	205,000 (400,000)* 87 12 5 mm.	46,000 46 4			
23 days after inoculation	White blood count Per cent of lymphocytes Per cent of lymphoblasts Size of lymph nodes	209,000 (500,000)* 89 9 5 mm.	158,000 — —			

** Spleen 2 cm. long.

* Estimated from smear.

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EXPLANATION OF PLATES

PLATE 23

FIG. 1. Lymph node in spontaneous lymphoid leucemia. The original architecture is obscured by a massive growth of lymphoid cells extending beyond the capsule of the lymph node (Mouse A 8). Hematoxylin, eosin and azure II. $\times 300$.

FIG. 2. Spontaneous lymphoid leucemia. Infiltrations in the liver (Mouse A 8). Hematoxylin and eosin. $\times 200$.

FIG. 3. Spontaneous lymphoid leucemia. Infiltrations in the kidney (Mouse A 8). Hematoxylin and eosin. $\times 200$.

FIG. 4. The blood smear in lymphoid leucemia produced by transmission. White blood count 691,000. Wright and Giemsa's blood stain. $\times 1000$.

PLATE 24

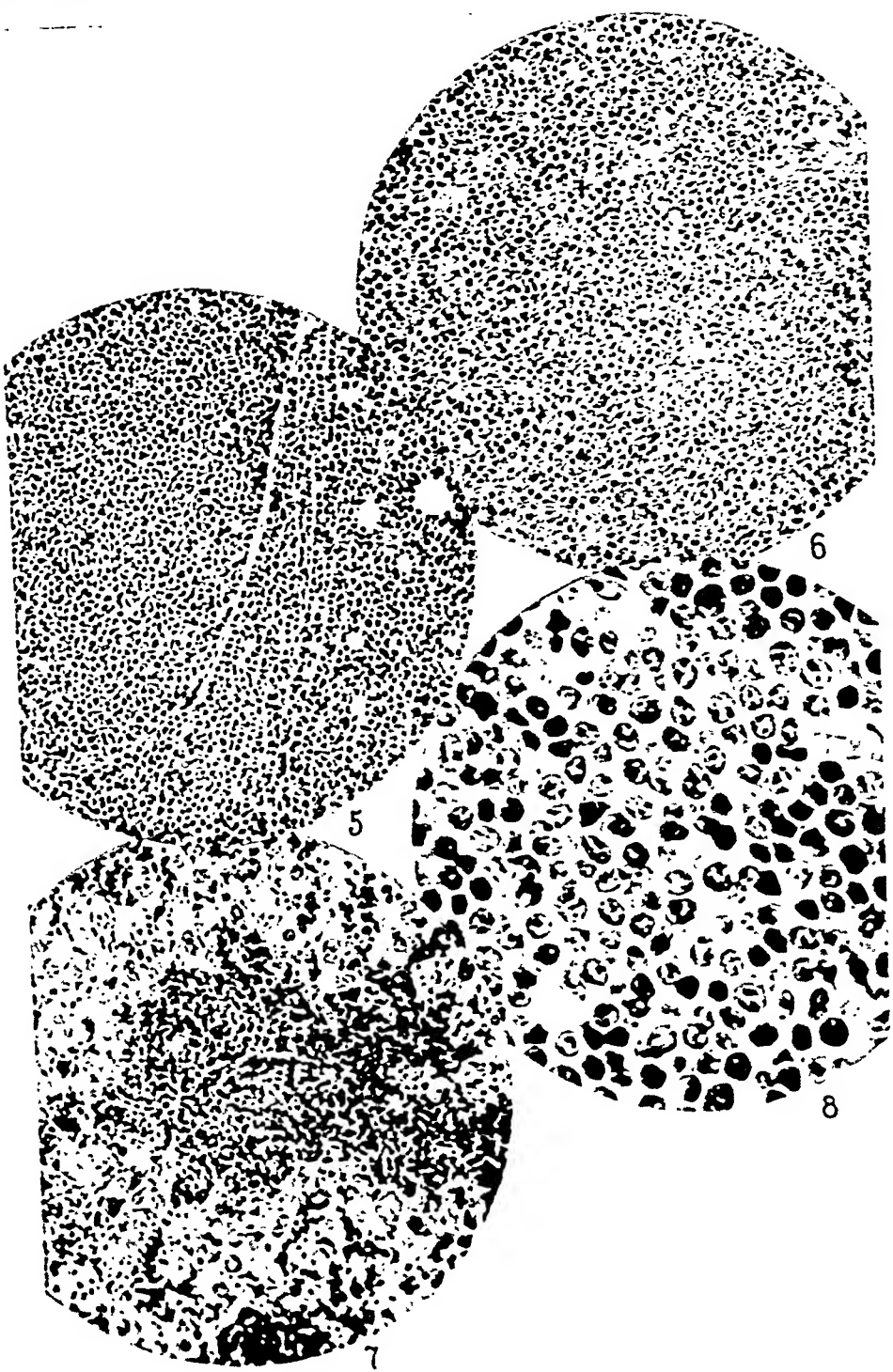
FIG. 5. The lymph node in lymphoid leucemia produced by transmission. There is a massive infiltration of the fatty tissue surrounding the lymph node. Hematoxylin and eosin. $\times 200$.

FIG. 6. The hyperplastic splenic follicle in transmitted leucemia of mice. Hematoxylin and eosin. $\times 200$.

FIG. 7. Lymphoid infiltration in the liver of a mouse with leucemia produced by transmission. Hematoxylin and eosin. $\times 200$.

FIG. 8. View of a lymphoid tumor produced by intraperitoneal inoculation in Mouse A 931. Hematoxylin and eosin. $\times 700$.





about 700 negroes and three or four whites. In addition, a squad of four marines lives on the beach at Cruz Bay (the port of the island) and runs the radio station. Such communication with the other islands as exists is by means of small sailboats; but there is no active commerce, and movement of population is very slight. A great many of the natives have never left the island, and virtually none of them have ever been out of the tropics. The negroes are descendants of former slaves and dwell in huts scattered about the mountains. They are indolent and very poor, living on fish, corn-meal, and a few tropical fruits and vegetables. Their nutrition is poor. One striking feature is the absence of communicable diseases. Scarlet fever and diphtheria are unknown. There have been some cases of measles among adults in years past, but none among children. Tuberculosis is not a major problem. Pneumonia and typhoid fever are rare. Hookworm disease is not prevalent, and there was no case of malaria during the year.

Colds were prevalent but very mild. We have divided the cases arbitrarily into four groups.

Group 1. Very Mild.—This group includes persons that had slight nasal discharge or slight cough for a few days, with no other symptoms.

Group 2. Mild.—This represents a group having nasal discharge, cough, perhaps generalized symptoms such as headache and boneache, but slight or no fever and no incapacity.

Group 3. Moderate.—This group includes persons with characteristic symptoms of a cold, a mild fever (100°F.) for a day or two, and incapacity for 3 or 4 days. None was confined to bed.

Group 4. Severe.—The final group, a small one of seven cases, includes those persons who were confined to bed for a day or more with fever of 101°F. or higher and who suffered all the characteristic symptoms of a bad cold. In this group would probably be found such cases as would be considered severe enough to require the services of a physician under the average social conditions of one of our large cities.

The incidence by months of colds in the environs of Cruz Bay is given in Table I. In a population of 223 persons, there were 184 colds in the course of a year. Over 80 per cent of these colds were mild, and only 3.8 per cent were severe. There were four cases of tonsillitis in the group. We believe that this cross-section of Cruz Bay gives a true picture of the incidence of respiratory disease on the whole island of St. John. We saw every case of severe cold in the entire population and most of the moderate and mild cases. No case of pneumonia occurred on the island during the year.

This census indicates that colds are not only less severe in St. John

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A BACTERIOLOGICAL STUDY OF "COLDS" ON AN ISOLATED TROPICAL ISLAND (ST. JOHN, UNITED STATES VIRGIN ISLANDS, WEST INDIES)

By DANIEL F. MILAM, M.D., AND WILSON G. SMILLIE, M.D.

(From Laboratories of The Rockefeller Institute for Medical Research, and the International Health Division of the Rockefeller Foundation, New York)

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The investigations here reported are part of a study of colds in isolated communities under different environmental conditions. The results of previous investigations in Alabama and Labrador have been published (1, 2, 3). This third field study was conducted in St. John, one of the Virgin Islands.

Our field laboratory was established at Cruz Bay on April 18, 1929, and observations were continued for 1 year. The same bacteriological technique that had been developed in the previous field studies was followed, but the epidemiological data and meteorological information were recorded in much greater detail and over a longer period. Records were kept of the daily maximum and minimum temperatures. Four hourly readings were made during the day, of relative humidity and barometric pressure; and daily rainfall was recorded.

Early in the work, our field staff was put in charge of all medical service on the island, and two small clinics were established where free advice and treatment were given. Through these clinics we obtained a general knowledge of all illness in St. John during the year. In addition, a group of 232 persons in the environs of Cruz Bay was selected for special study. By frequent regular house-to-house visits to these persons a fairly accurate census was obtained of all cases of acute colds which occurred.

Typography and Type of Population Incidence of "Colds"

St. John, which is typical of the smaller islands of the West Indian group, is mountainous and about 12 miles in length. It has a permanent population of

"COLDS" ON AN ISOLATED TROPICAL ISLAND

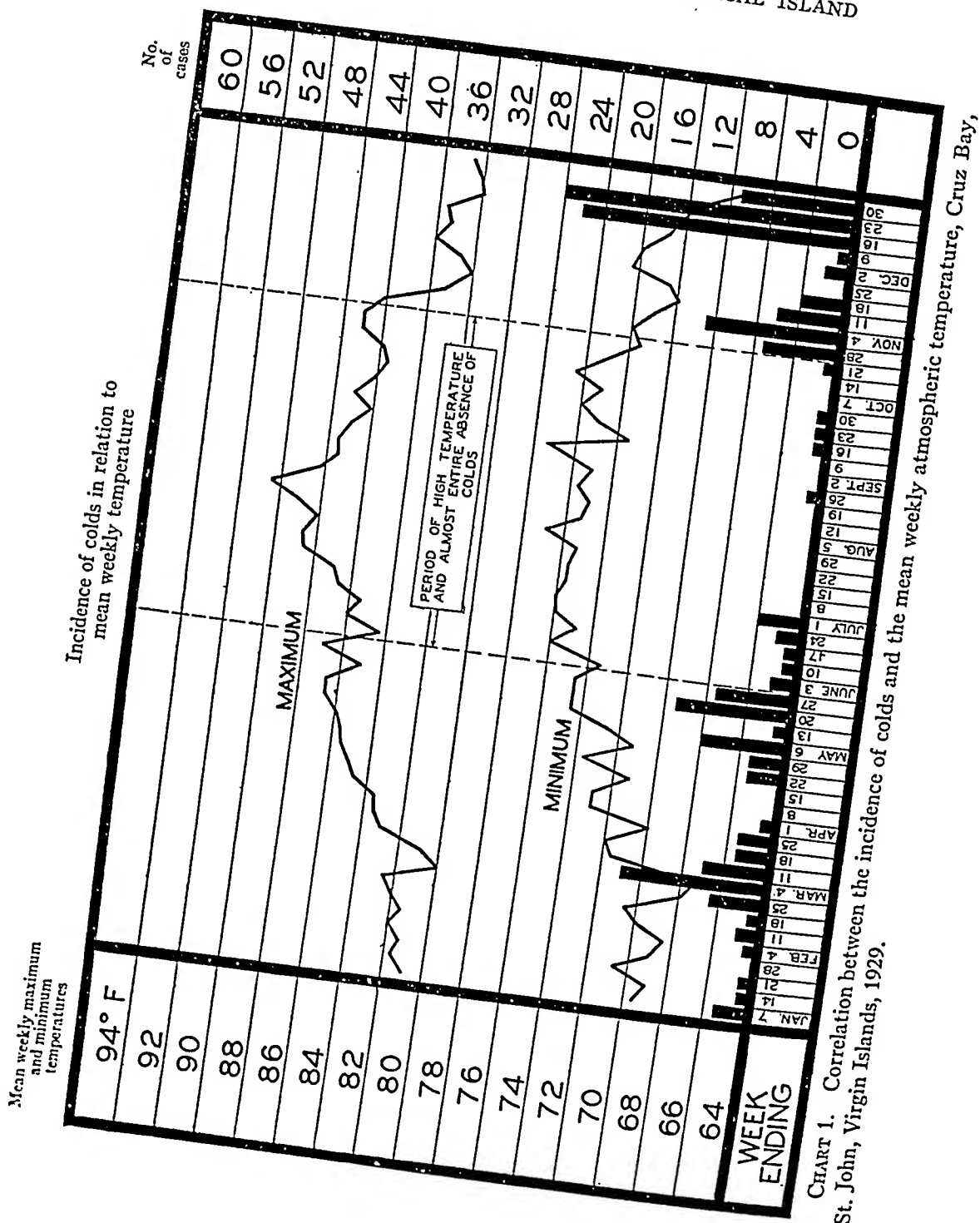


CHART 1. Correlation between the incidence of colds and the mean weekly atmospheric temperature, Cruz Bay, St. John, Virgin Islands, 1929.

than in temperate climates but much less prevalent. The comparable data of Townsend and Sydenstricker (4), Jordan (5) and his co-workers in the United States, and Van Loghem (6) in Holland showed an average of over two colds per person per year in the temperate zone, with a higher percentage of severe cases than occurred in St. John.

The correlation between the incidence of colds in Cruz Bay throughout the year and variations in atmospheric temperature, is given in Chart 1. Mean maximum and minimum weekly temperatures have

TABLE I
Incidence of Colds in Cruz Bay, St. John, 1929-30

	Very mild	Mild	Moderate	Severe	Total
April.....	6	1	3	0	10
May.....	8	12	1	2	23
June.....	4	4	2	0	10
July.....	0	0	0	0	0
Aug.....	0	0	1	0	1
Sept.....	0	2	1	0	3
Oct.....	2	7	2	1	12
Nov.....	6	7	4	1	18
Dec.....	30	25	8	2	65
Jan.....	1	3	1	1	6
Feb.....	10	5	0	0	15
March.....	12	6	3	0	21
Year.....	79	72	26	7	184

been plotted on the chart. In order to show seasonal variation the data are recorded from January 1 to December 31. A striking feature of the temperature records is their constancy. Throughout the year there is a drop in temperature each day, between midafternoon and midnight, of about 12°F., so that the daily maximum and minimum temperature records are two parallel lines about 12° apart. There is a variation of only 12° to 15°F. in daily maximum temperature throughout the whole year. In fact, the difference between the morning and evening temperature of each day is as great as the difference between the temperature of summer and winter.

Nevertheless, though seasonal variations in temperature are

forced to conclude that the colds had a common source, probably infectious in nature, spread by direct contact and with a definite incubation period, though it seems most probable that environmental factors, such as relatively low temperature, predisposed to infection.

We noted no correlation between changes in barometric pressure, changes in relative humidity, or total rainfall, and the incidence of colds. The people themselves were convinced that rapid cooling of the body surface such as occurred if they were caught in a night rain when the wind was blowing, or if they worked hard and then cooled their perspiring bodies quickly in the wind, would certainly result in a cold. Our observations give no evidence as to whether or not this factor is of importance in incidence of colds.

Bacteriological Findings

Technique.—The bacteriological methods employed in studying the nasopharyngeal flora in St. John were the same as those used in the Alabama and Labrador studies and have already been reported (2); it is unnecessary, therefore, to describe them here. Hormone broth was the basic medium, enriched with rabbit blood. The methods followed in previous studies for identification of the various organisms and the system of recording results were continued in this study.

During the year, nasopharyngeal cultures were made upon almost half the population of the island. At first, normal individuals of all ages were selected at random for this purpose. At the Cruz Bay school, which had an enrollment of 75 children from 6 to 15 years of age, cultures were made on all pupils each quarter-year. Repeated cultures were made on a smaller group in the environs of Cruz Bay. In addition repeated cultures were made on the small white population, including the laboratory personnel.

Attempts were made to take cultures from every person with an acute cold as early and as often as possible. A total of 831 throat cultures, taken from 385 persons, were analyzed. The data were analyzed by age, sex, and color groups, by quarter-year, and in relation to illness. There was no significant variation between adults and children or between the sexes, so these data have been grouped. The seasons of the year blend so gradually, with no real spring or fall, that it seemed best to group all the bacteriological data into two chief

slight, there is a correlation between incidence of colds and season. Practically no colds occurred from the end of May to the latter part of October (see Chart 1). During this period the daily maximum temperature was above 86° and the daily minimum temperature above 74° . Late in October the temperature dropped about 4° . Coincidentally a small outbreak of colds occurred. In December the temperature fell consistently each night to 70°F. or slightly below, and at this period there occurred a sharp outbreak of colds. This was followed by a third outbreak in March, with scattered cases appearing through April and May. Townsend and Sydenstricker (4) and many other observers, including Van Loghem (6) in Holland, have noted this periodicity of colds, with a peak in the fall, a decline, and a second higher peak in the late winter. The seasonal incidence and duration of the outbreaks in St. John are very like those recorded by observers in temperate zones, but the total number of cases in the tropics is much less than in the temperate zones.

There is some evidence that the sharp outbreak in December (see Chart 1) was introduced by a sailor on the mail boat who may have become infected in St. Thomas, the cosmopolitan center of this group of islands. His was the first case in the outbreak. Secondary cases appeared in his family and among his immediate neighbors in Cruz Bay. The disease then spread rapidly in concentric circles up over the mountains to the surrounding settlements, sometimes invading a whole family simultaneously, but more often affecting only one member of a family at first. Then, after 2 to 3 days, other members of the family developed the disease. Thus we have excellent evidence to indicate that this outbreak was spread through direct contact of well persons with an infected individual, the incubation period being 2 to 3 days in each case.

We have evidence also that these colds were due to change in temperature. There was a distinct drop in the temperature early in December, and colds occurred coincidentally with the decline in minimum night temperature (see Chart 1). If the disease had been initiated solely by environmental factors, one would have expected that all individuals in all the communities would have developed the disease simultaneously. Since the disease spread in concentric waves from a central point, with definite periods of time intervening, we are

organisms found in the throats of normal negroes in summer in St. John were staphylococci. (See Table II.)

But some persons may have small numbers of a given organism in their nasopharynx, while in others this strain may predominate. To bring out this point, we have grouped all individuals into five subdivisions, in accordance with the prevalence of each organism in their throats.

In studying the prevalence of Gram-negative diplococci, for example, the individual throat cultures are divided as follows:

- Individual incidence of Gram-negative diplococci in the nasopharynx:
- Group 1. None (no Gram-negative diplococci in their plate culture).
- Group 2. Few (1 to 25 per cent of all organisms on the plate culture).
- Group 3. Moderate (26 to 50 per cent of all organisms on the plate culture).
- Group 4. Many (51 to 75 per cent of all organisms on the plate culture).
- Group 5. Very many (76 to 100 per cent of all organisms on the plate culture).

These data are called *individual incidence* of organisms. The *bacterial group incidence* and the *individual incidence* of Gram-negative cocci, both in winter and summer, in normal persons and in those with colds are plotted in Chart 2. Similar data are given for staphylococci and Pfeiffer's bacilli on Chart 2; indifferent streptococci, hemolytic streptococci, and green streptococci (*viridans* group) on Chart 3; pneumococci, "intermediates," and "others" on Chart 4.

The intermediates are described in our previous publications. They represent that group of organisms that resembles both pneumococci and *St. viridans*. They may be bile-soluble or partially so, and may or may not ferment inulin. The organisms classified as "others" are of wide variety and are found in the throat in small numbers, or occasionally in large numbers. Included are the diphtheroids, various types of Friedländer's bacilli, and many unidentified strains.

The most striking feature of the bacteriological data is the constancy of the findings. There was some individual variation, but in so far as our data went, we were unable to determine any significant variation in the nasopharyngeal flora in persons with colds. The colds were more prevalent in the winter months, and certain organisms were more prevalent in winter than in summer, but the increase in these organisms occurred in normal throats as well as in those of persons with colds.

seasonal divisions, winter (October 15 to April 14) and summer (April 15 to October 14).¹

The bacterial group incidence in normal persons is compared with the results obtained from persons with acute colds. A summary of the year's bacteriological data is given in Table II. The *bacterial group incidence* is an index of the prevalence of any given type of

TABLE II

Bacterial Group Incidence of Nasopharyngeal Flora in Normal Persons and in Persons with Colds, in Summer and Winter, St. John, Virgin Islands

(The mean with the standard deviation is given in each instance)

Group	Number of cultures	Gram-negative cocci	Staphylococci	Pfeiffer's bacillus	Indifferent streptococci	Hemolytic streptococci	Green streptococci	Intermediates	Pneumococci	"Others"
Normal colored persons, summer	315	10.0 ±.7	11.4 ±1.0	13.0 ±1.0	10.9 ±.8	4.0 ±.7	23.1 ±1.3	5.8 ±.8	14.3 ±1.0	10.3 ±1.0
Normal colored persons, winter	231	8.7 ±.8	6.2 ±1.0	10.6 ±.9	12.6 ±.9	1.7 ±.4	26.8 ±1.6	10.5 ±1.2	13.8 ±1.4	8.8 ±1.1
Colored persons with colds, summer	78	12.8 ±1.6	8.9 ±1.9	10.3 ±1.5	14.4 ±1.9	2.5 ±1.1	25.0 ±2.5	3.5 ±1.2	13.6 ±2.0	8.7 ±2.3
Colored persons with colds, winter	70	9.4 ±1.6	4.5 ±1.6	7.9 ±1.4	11.5 ±1.6	1.5 ±1.1	29.5 ±3.0	13.9 ±2.8	15.4 ±2.9	6.4 ±1.6

organism in the population. It may be explained by the following illustration: If we imagine that all cultures were taken from one composite nasopharynx and planted upon one gigantic blood agar plate, then the percentage of organisms of each type found on this plate would represent the bacterial group incidence of that particular organism in the community. For example, 11.4 per cent of all

¹ Detailed analyses of the bacteriological data by quarters are given in the official report to the Rockefeller Foundation and are available to any one who may wish to consult them.

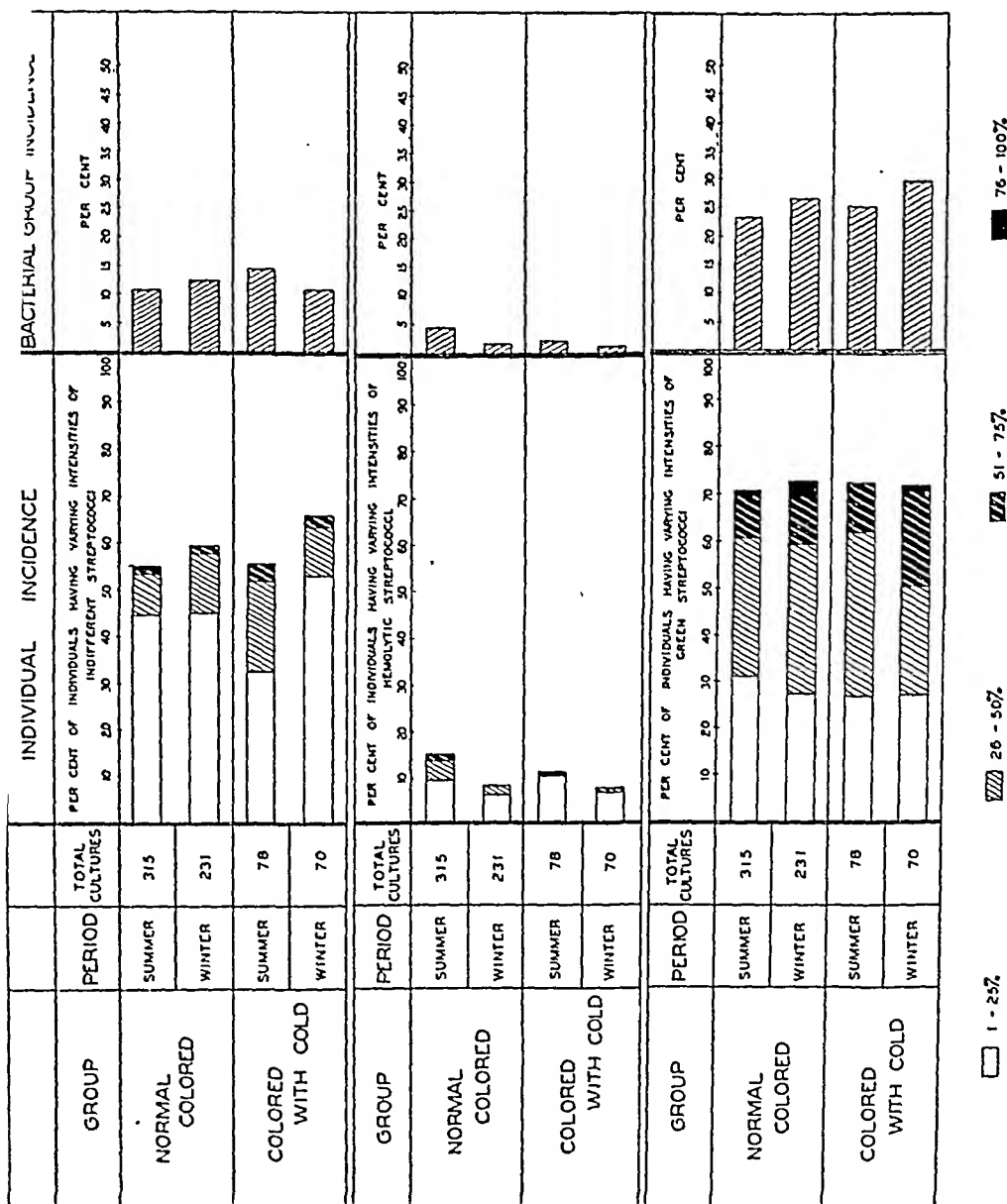


CHART 3. Incidence of indifferent streptococci, hemolytic streptococci, and green streptococci in the nasopharyngeal flora of normal colored persons and of those with colds, summer and winter, St. John, Virgin Islands.

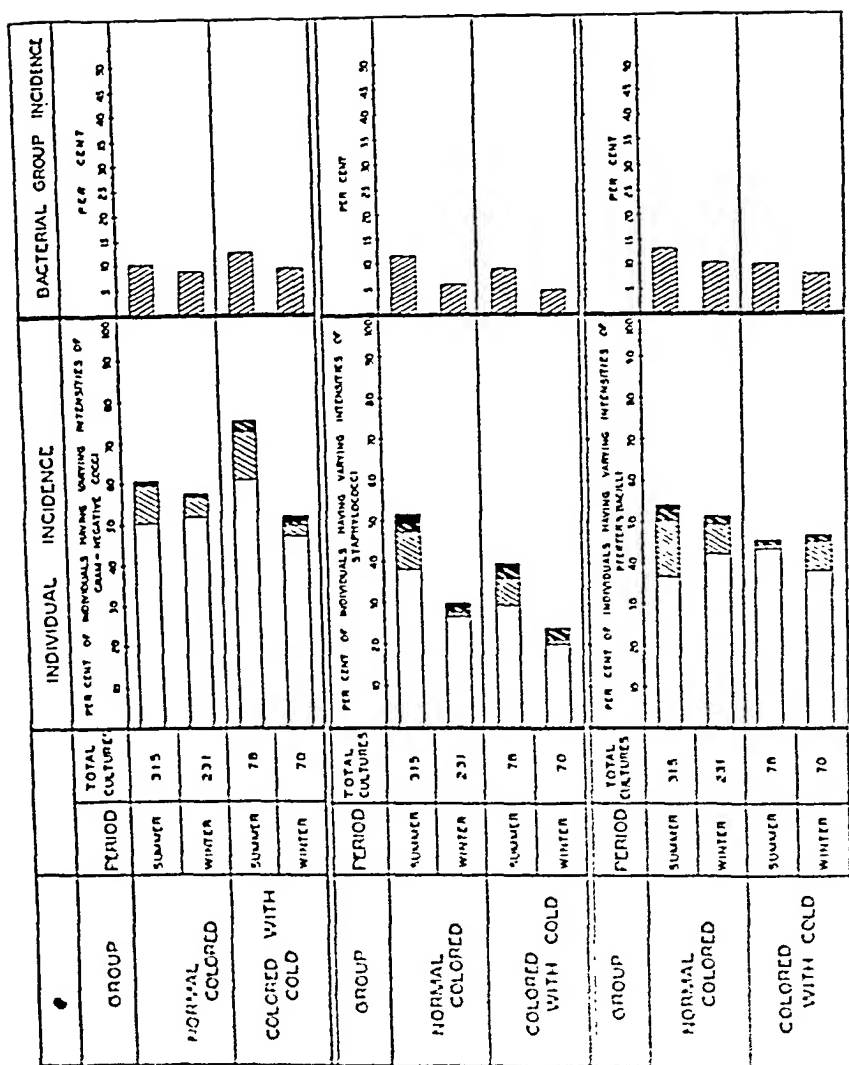


CHART 2. Incidence of Gram-negative cocci, staphylococci, and Pfeiffer's bacilli in the nasopharyngeal flora of normal colored persons and of those with colds, summer and winter, St. John, Virgin Islands.

Gram-Negative Cocci.—From 9 to 12 per cent of all organisms present were Gram-negative diplococci. Their prevalence remained quite constant throughout the year, in normal persons as well as in those with colds. No meningococci were found.

Staphylococci.—In the negroes, staphylococci were more prevalent in the summer months than in the winter. In the small white population they were much more prevalent in winter, but there was no relationship between the increased prevalence and respiratory disease. Almost one-half the staphylococci were

TABLE III

**Classification of Pfeiffer's Bacillus for Two Seasons, in Persons with and without Colds, St. John, Virgin Islands*

Group			Non-hemolytic				Hemolytic				Unde- termined		Total
			True <i>B. pfeifferi</i>		<i>Para-influenzae</i>		Influenza		<i>Para-influenzae</i>		Hemolytic	Non-hemolytic	
	Period	Total cultures	Indol +	Indol neg.	Indol +	Indol neg.	Indol +	Indol neg.	Indol +	Indol neg.			
Normal colored persons	Summer	315	3	13	3	45	1	17	2	81	10	5	180
Normal colored persons	Winter	231	—	1	5	23	1	4	5	87	—	—	126
Colored persons with colds	Summer	78	—	6	—	17	—	4	1	11	4	—	43
Colored persons with colds	Winter	70	—	—	—	9	—	—	—	24	—	—	33
Year.....		694	3	20	8	94	2	25	8	203	14	5	382
Year, per cent..			.8	5.2	2.1	24.6	.5	6.5	2.1	53.2	3.7	1.3	100

* Rivers' classification of Pfeiffer bacilli is used in this table.

S. aureus, and a large proportion of these *S. aureus* strains were hemolytic. About one-third of the *S. albus* strains were also hemolytic.

Pfeiffer's Bacilli.—These organisms were prevalent (8 to 12 per cent of all organisms cultivated). They were encountered more often in the summer than in the winter. The strains were subdivided according to Rivers' classification.

Table III summarizes the results of our findings in the influenza group. The great proportion are hemolytic *para-influenzae* strains that do not form indol. Only 23 of the 382 strains isolated were true Pfeiffer's bacilli, and only three of

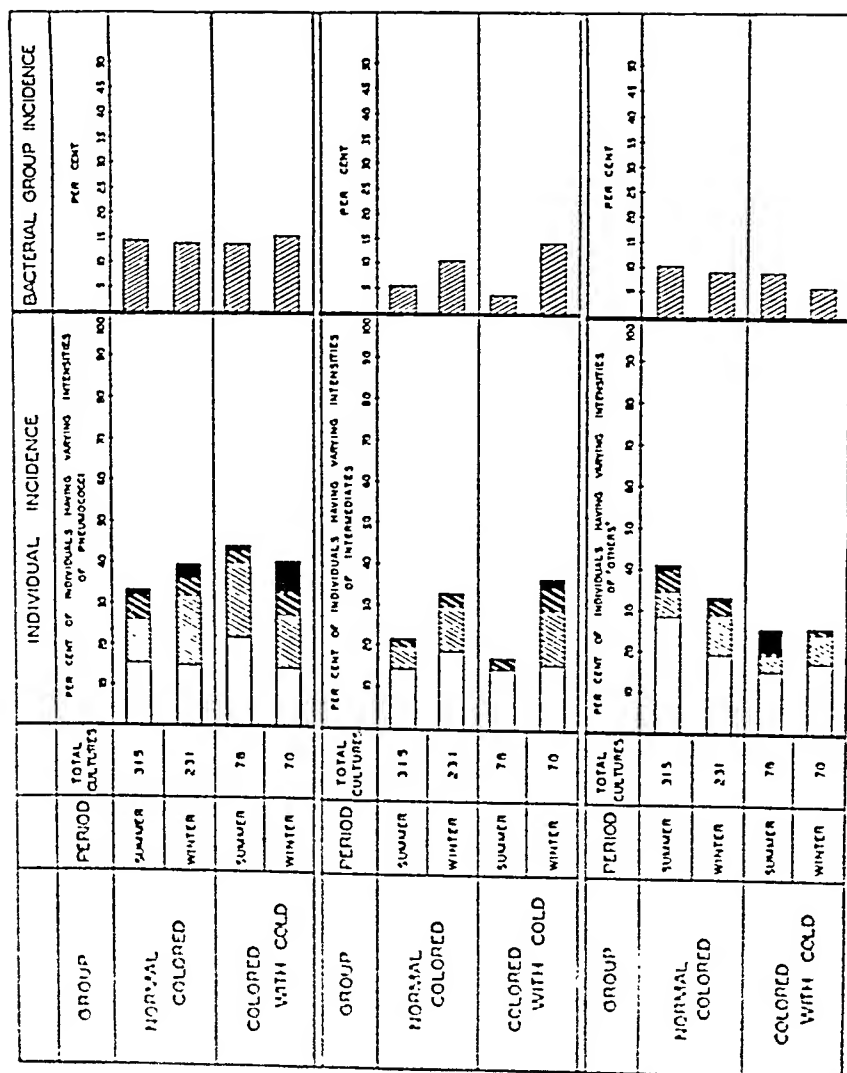


CHART 1. Incidence of pneumococci, intermediates, and "other organisms" in the nasopharyngeal flora of normal colored persons and of those with colds, winter and summer, St. John, Virgin Islands.

Other Organisms.—There are many organisms which were found in the nasopharynx only occasionally, and usually in small numbers. They made up about 5 to 10 per cent of the total flora. The most common group includes the diphtheroids. The diphtheria bacillus was found only once, and the strain was avirulent. No clinical diphtheria has been reported from St. John in many years. In order to determine the degree of immunity of the inhabitants to diphtheria, Schick tests were made on 97 children between 5 and 15 years of age. Only nine gave positive Schick tests. Two were over 9 years of age and seven under 9 years. This high degree of communal immunity to diphtheria in St. John is in accordance with the findings of Doull (8) in Brazil, who suggests that infection with the diphtheria bacillus in the tropics is widespread but seldom rises to clinical significance. We are unable to explain the high degree of immunity to diphtheria in St. John.

TABLE IV

Bacterial Group Incidence of Nasopharyngeal Flora in Normal White Persons and in Those with Colds, Summer and Winter (12 Months), St. John, Virgin Islands

(The mean and the standard deviation are given in each instance)

Group	Period	Total cultures	Gram-negative cocci	Staphylococci	Pfeiffer's bacillus	Indifferent streptococci	Hemolytic streptococci	Green streptococci	Intermediates	Pneumococci	"Others"
Normal	Summer	49	10.4 ±2.1	10.1 ±3.0	9.1 ±2.3	26.1 ±4.1	.5 ±.3	16.9 ±2.6	3.7 ±1.3	16.0 ±3.7	7.0 ±1.9
Normal	Winter	33	12.2 ±2.1	20.3 ±5.7	7.1 ±2.5	27.7 ±3.8	.4 ±.3	21.7 ±3.4	4.9 ±2.1	1.7 ±1.6	2.4 ±1.0
With cold	Summer	8	15.0 ±4.7	7.5 ±1.9	2.5 ±1.4	13.8 ±4.1	1.3 ±1.1	40.6 ±7.7	10.6 ±4.9	.6 ±.2	8.1 ±5.4
With cold	Winter	11	12.3 ±3.8	17.3 ±8.7	20.0 ±6.4	13.6 ±7.2	2.7 ±1.8	26.8 ±5.7	0 0	5.0 ±2.9	6.8 ±3.8

Friedländer's bacillus was found quite frequently in the negroes (fifty-four times), but only once in the whites. It was much more prevalent in the tropics than in the other areas that we have studied, but did not seem to be associated with any nasopharyngeal disturbance.

Nasopharyngeal Flora of the White Group.—A summary of the bacteriological findings in the small group of white persons is given in Table IV. There were no marked differences from the findings in the negroes.

these formed indol. No relation was observed between the increased prevalence of any of the strains of Pfeiffer's bacilli and the incidence of colds.

Indifferent Streptococci.—This group comprised from 11 to 14 per cent of all organisms found. There was little or no variation throughout the seasons.

Hemolytic Streptococci.—Organisms of this group were found infrequently, and usually when their presence was noted on the plate they were few in number. About 3 per cent of all organisms encountered were true beta hemolytic streptococci. They were slightly more prevalent in summer than in winter. When the group is divided into subgroups, we note that though hemolytic streptococci were encountered 93 times, only 37 of these strains were true *Streptococcus pyogenes*, the remainder being *St. equi*, *St. alactosis*, etc. There was no apparent relationship between incidence of colds and the appearance of *St. pyogenes*.

St. viridans.—The prevalence of the green streptococci was strikingly constant throughout the year. They were found in nearly every throat, comprising about 25 per cent of the total flora. There was no variation with the seasons, nor in persons with colds. The group was subdivided according to carbohydrate fermentations. *St. salivarius*, *St. mitis*, and *St. equinus* were most frequently encountered, often all were present in the same throat. These strains are apparently normal and more or less constant inhabitants of the nasopharynx.

Pneumococci.—From 13 to 15 per cent of all organisms found were pneumococci. They were found in about 40 per cent of all throats from which cultures were taken and were no more prevalent in winter than in summer. No increase occurred in persons with colds.

The strains were subdivided in accordance with Cooper's (7) classification. Types I, II, and III are the same as Cole's original classification; but Group IV has been divided further. Dr. Cooper kindly furnished us with type-specific sera for Types IV to XIII inclusive and also type serum for Nos. XV and XVIII.

Types I and II pneumococci were never found. Type III was found eight times, first in the throat of one of us, and it is possible that we may have introduced this strain. The remaining 251 identified strains all fell into Group IV; of these 55 were agglutinated by some one of the Cooper sera. These strains were widely distributed, each Cooper type being encountered at least once. Most of the strains had little or no virulence. (Our standard test was an intraperitoneal inoculation into white mice of 0.1 cc. to 0.5 cc. of a 1:10 dilution of an 18 hour hormone broth culture.) The strains that were virulent to white mice were not more prevalent in persons with colds than in normal throats, and were as prevalent in summer as in winter.

Intermediates.—The intermediates are organisms that resemble both *St. viridans* and pneumococci. The colony formation and morphology is such as to suggest pneumococci, and they usually ferment inulin. As a rule they are bile-soluble. Those strains were much more prevalent in the winter months (about 12 per cent of all organisms) than in the summer, when only 4 per cent of the organisms fell into this class. They were not more prevalent in the sick; the increase in winter occurred in normal throats as well as in those with respiratory disease. We have no explanation of the significance of this observation.

only three of these strains produced indol. In Labrador and Alabama, hemolytic strains of influenza bacilli were much less prevalent than non-hemolytic strains. In both Labrador and Alabama, epidemics of colds were associated with a marked increase of true Pfeiffer's bacilli, which required both V and X substance for growth and for the most part produced indol. This phenomenon did not occur in St. John.

Indifferent Streptococci.—These were found infrequently in Alabama. In St. John they were about as prevalent as in Labrador. The group is apparently a normal and fairly constant inhabitant of the human throat.

Hemolytic Streptococci (Beta Type).—Hemolytic streptococci were rarely found in the Virgin Island study. Only 3 per cent of the people harbored true *St. pyogenes*, and when found, the organisms were few in number. This was in accordance with the findings in Alabama and Labrador, and apparently is a characteristic of isolated populations. It is interesting to note in this connection that in St. John scarlet fever is unknown, tonsillitis is very infrequent, and other infections associated with hemolytic streptococci, such as erysipelas, puerperal sepsis, etc., are infrequent. It is our belief that *St. pyogenes* is essentially an organism of civilization and industrial overcrowding.

The Viridans Group (Alpha Hemolytic Streptococci).—This group of organisms was slightly more prevalent in Alabama than in St. John and somewhat less prevalent in Labrador. Their presence was constant in the tropics (about 25 per cent of all organisms cultivated). These strains are apparently part of the normal basic flora of the nasopharynx of mankind.

Intermediates.—These strains were commonly found in Alabama and Labrador in the course of epidemics of colds. In St. John they were twice as prevalent in the winter as in the summer months, the bacterial group incidence increasing from about 5 per cent to 10 per cent, but the increase was just as great in the normal throats as in those of persons with colds. We do not know the significance of this variation.

Pneumococci.—In Alabama there was an increased prevalence of pneumococci during an epidemic of colds, with almost entire absence of these organisms in normal periods. Some of the strains were highly virulent for white mice, and cases of pneumonia were frequent during the epidemic of colds that swept over the whole countryside.

In Labrador there was a high prevalence of pneumococci in normal throats. In fact 45 per cent of all organisms isolated from the Indian nomads were pneumococci. These organisms did not increase during an epidemic of colds.

In the Virgin Islands the bacterial group incidence of pneumococci was strikingly constant (from 13 to 15 per cent of all organisms isolated). They were no more prevalent in winter than in summer, nor did they increase in persons with colds.

In all three communities the incidence of fixed virulent types of pneumococci, i.e., Types I, II, and III, was very low. A few Type I strains were found in

Staphylococci were not prevalent in our own staff on arrival in the tropics but these organisms soon became even more prevalent in us than in the negroes. One of the staff harbored Type III pneumococcus early in the work, but it soon disappeared. Pfeiffer's bacilli increased in the whites in a few instances in the winter colds, and pneumococci were less prevalent in the whites than in the blacks.

The incidence of colds in the white inhabitants of the island was much lower than these persons had experienced in the temperate zone, and the attacks were so mild as to be almost unnoticeable. Only one white person had a severe cold. This occurred during the December epidemic (see Chart 1) and was apparently contracted through contact with a sick negro servant.

Comparison of Bacteriological Findings in the Tropics with Findings in Labrador and Alabama

In general, the same types of organisms were found in the nasopharynx of persons living in isolated communities in the tropics, in the temperate zone, and in the Arctic zone. Certain differences were noted which are of interest and perhaps may be of importance.

Gram-Negative Cocci.—Organisms of this group were slightly less prevalent in the tropics than in Labrador and Alabama. In none of the areas studied did they seem to be of pathological significance; but they are a part of the normal flora. Meningococci were not found in any area studied.

Staphylococci.—Staphylococci, particularly the hemolytic types, were much more prevalent in the tropics. This was true not only in the case of negroes but of whites as well. Hemolytic staphylococci apparently became implanted in the throats of our laboratory workers, and in one individual this organism was found constantly in large numbers.²

Pfeiffer's Bacilli.—The group of Pfeiffer's bacilli was quite as prevalent in the Virgin Islands as in Labrador and Alabama. If we divide these strains into subgroups we find striking differences. The most prevalent type in St. John was a hemolytic, *para-influenzae* strain, requiring only V substance for growth and producing no indol. True Pfeiffer's bacilli, non-hemolytic and requiring both V and X substance for growth, totalled only 23 out of the 382 strains isolated, and

² The Institute of Tropical Medicine in Porto Rico also reports a high incidence of hemolytic staphylococci in the nasopharynx of the people in that island (personal communication). We are unable to interpret the significance of these findings.

were most prevalent from the 4th to the 7th day of the infection. These observations suggest that colds in all three areas were initiated by some factor which we have not yet discovered, and also indicate that colds may run a mild course without any modification of the customary nasopharyngeal picture. It seems probable, however, that the severe secondary symptoms associated with colds in Labrador and Alabama were produced by organisms with which we are perfectly familiar, namely, pneumococci and Pfeiffer's bacilli.

Is the inciting etiological factor of colds an environmental one or a specific infectious agent? Epidemiological evidence from all three areas seems to indicate that it is infectious, and spread by direct contact, with an incubation period of 1 to 3 days. Nevertheless, the evidence is very strong, particularly in Alabama and St. John, that environmental factors play a definite part in the incidence of colds. In each instance, epidemics of colds followed a drop in atmospheric temperature. Furthermore, the seasonal curve of incidence of colds in the tropics is very similar to the seasonal curve in the temperate zone. The differences are of degree only. In the temperate zone, changes in seasonal temperature are more abrupt and colds are more severe; in the tropics, the changes in temperature are mild and colds are mild. Certainly, then, we cannot rule out environmental influences as at least predisposing if not inciting factors in the production of acute colds.

Streptococcus pyogenes was rarely encountered in the nasopharynx in the three isolated communities which we studied. Infections commonly associated with this organism, such as scarlet fever, erysipelas, puerperal sepsis, etc., were rare in these areas. We believe that absence of these diseases is due not to natural or acquired immunity but to absence of virulent hemolytic streptococci in the community.

Diphtheria bacilli were rare in St. John, being found only once, and clinical diphtheria was unknown. Nevertheless the children showed a very high immunity to diphtheria as indicated by the Schick test, far higher than is found in temperate zones where clinical diphtheria is prevalent.

We have noted the high prevalence of hemolytic staphylococci in the tropics, but have no explanation for it. Nor do we know the significance of the relatively high prevalence of Friedländer's bacillus.

Alabama and a few Type III strains in all three areas. Type II was never encountered. The great majority of the strains fall into Group IV. In Labrador and St. John these strains had a low virulence for white mice. Correspondingly, pneumonia was rare in both areas. In Alabama some of the Group IV strains had a high virulence for mice, particularly those strains isolated from actual cases of pneumonia.

Filter-Passing Anaerobic Bacteria.—In addition to the studies of aerobic flora of the nasopharynx, cultures were made of the filter-passing anaerobic groups of bacteria in the nasopharynx in normal persons and in individuals ill with colds. The technique followed was that devised by Olitsky and Gates (9) and Olitsky and McCartney (10). A report of these findings will be given in a separate paper.

DISCUSSION

Two arresting facts were noted in the Virgin Island studies: first, the mild type of colds that were encountered; second, the constancy and uniformity of the bacteriological flora of the nasopharynx.

If we consider the previous studies in Labrador and Alabama in conjunction with the St. John studies, we note that certain flora of the nasopharynx are found consistently in uniform numbers in all areas. In these bacterial groups are included Gram-negative diplococci, the *viridans* group of streptococci, the indifferent streptococci, and the diphtheroids. These organisms are apparently normal basic flora and are not related to acute respiratory disease. It is possible, of course, that with more refined methods there would be detected certain subtypes within these general groupings that are of pathological significance.

Pneumococci did seem to have some pathological significance in an epidemic of colds in Alabama. In Labrador, an epidemic of colds was associated with an increase in influenza bacilli, but pneumococci, which were prevalent in normal throats, did not increase during the epidemic. In the tropics, on the other hand, neither pneumococci nor influenza bacilli increased in numbers during an outbreak of colds. We have no evidence from our St. John data that any of the aerobic flora commonly encountered in the nasopharynx played a part in the initiation or course of colds.

The colds in St. John were mild, whereas those in Labrador and in Alabama were severe, the latter particularly so. Furthermore, the increase in influenza bacilli in Labrador and in pneumococci in Alabama did not occur during the initiatory stages of the disease, but

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Neither organism seemed to play any part in increased prevalence of disease.

SUMMARY

Studies in the Virgin Islands, Labrador, and Alabama, suggest that colds are incited by some specific agent with which we are not yet familiar. They suggest also that the secondary and more severe symptoms associated with colds may be due to certain aerobic flora commonly found in the nasopharynx. Types of pneumococci which are virulent (to white mice) and true Pfeiffer's bacilli requiring both V and X substance (and forming indol?) seem to be of particular importance in these secondary infections.

The studies indicate that the specific agent which initiates colds is infectious in nature, and spread by direct contact, with an incubation period of 1 to 3 days. There is strong evidence that environmental factors, particularly reduction in atmospheric temperature, have some influence upon the incidence of colds.

CONCLUSIONS

1. The results of a year's field study in St. John, United States Virgin Islands, West Indies, indicate that colds are less common and much less severe in the tropics than in the temperate zone.

2. The basic nasopharyngeal flora in normal persons in St. John is similar to that of normal persons in Labrador and Alabama. Pfeiffer's bacilli are much less common in St. John than in the temperate zone. Hemolytic staphylococci are prevalent in the tropics, hemolytic streptococci are rare. Pneumococci are prevalent in St. John but are avirulent. Fixed Types I, II, and III are rare.

3. The basic nasopharyngeal flora in St. John was quite constant throughout all seasons and in all groups of people. No change occurred in the nasopharyngeal flora in a group of persons who developed colds.

4. The seasonal incidence curve of acute colds in St. John was a replica in miniature of the same curve for the United States. No colds occurred during the very warm period from June to October. An epidemic of colds in December was coincident with a slight but abrupt drop in atmospheric temperature.

cation of the clinical forms of the disease, the stages in the experimental conditions correspond to Type I and Type IIa.

MacCallan's classification of conjunctival involvement, based on extensive experience with trachoma in Egypt and in England, is as follows:

Type I, usually seen soon after infection, is characterized by the presence in the conjunctiva of follicles chiefly of the tiny, grey, pin-head type, sparsely scattered, and more prominent on the upper membrane.

Type II is a later development and has three subtypes. In (a) there is predominance of enlarged follicles. In (b) papillary hypertrophy may exist with the follicles. Red, raspberry-like papillae, or elevations, mask the typical gelatinous follicles and occur more markedly on the upper conjunctiva. In still another subtype (c), also known as florid trachoma, the disease is characterized by a dark red, tumefied, velvety appearance of the conjunctiva, accompanied by mucopurulent discharges. In some cases the individual follicles can no longer be distinguished, becoming merged into a general infiltration. The condition may subside spontaneously or persist for several months if untreated.

Type III represents the stage in which cicatrization has begun and Type IV, also known as "cured trachoma," that in which scarring is complete.

Inasmuch as in the experimental disease, no corneal lesions have as yet been produced, those changes which deal with corneal involvement are not here described.

Through the cooperation of several ophthalmologists,¹ nine patients with trachoma have been available for study. The excised conjunctival tissue or expressed follicles or secretions of these patients showed by culture and in stained film preparations a large number and variety of bacteria. *Staphylococcus albus*, diphtheroids, *Bacillus xerosis*, and a Gram-negative chromogenic bacillus predominated.

In only seven of the nine cases did we obtain material sufficient in amount and fresh enough for cultivation experiments to disclose the presence of *Bacterium granulosis*. Of the seven, four yielded pathogenic cultures of *Bacterium granulosis* and the conjunctival tissue of these four, inoculated into monkeys, produced in each instance characteristic experimental granular conjunctivitis. Cultures were made in turn from the conjunctival tissue of two of the monkeys so affected and *Bacterium granulosis* was recovered in both instances. The conjunctival tissue from three patients, which failed to yield growths of the microorganism, was also injected into two or three monkeys in each instance. The tissue from one of these patients induced the characteristic granular conjunctivitis; cultures from one of the monkeys thus affected yielded no growth of *Bacterium granulosis*.

¹ We are deeply indebted for material to Drs. Martin Cohen, Arnold Knapp, Ervin Torok, and Julius Wolff, all of New York City.

THE EFFECT OF SECONDARY INFECTIONS ON EXPERIMENTAL TRACHOMA*

By P. K. OLITSKY, M.D., R. E. KNUTTL, M.D., AND J. R. TYLER

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 25 AND 26

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If it were possible to produce in monkeys the florid type of human trachoma commonly seen in practice, the evidence of the relation of experimental conjunctivitis to trachoma would be more convincing. The experiments to be reported here concern the rôle played by secondary infections in the experimental disease and particularly in the reproduction of the florid type of the disease in animals.

In this connection, we may cite an early observation by Muttermilch (1) (1909), based on clinical experience. He believes that trachoma is a mild disease and not dangerous to eyesight if the affection remains uncomplicated; that follicle and pannus formation are only complications and are not related to the causal agent; and that these distressing sequelæ result from infection by many different bacteria.

Noguchi (2) and others (3, 4, 5, 6) have described the conjunctival lesions induced in *Macacus rhesus* monkeys and chimpanzees by the inoculation of *Bacterium granulosis* as closely resembling those of trachoma in man. These authors have also shown that tissues derived from cases of human trachoma or from monkeys having the experimental disease induce, on conjunctival inoculation in animals essentially the same clinical and pathological effects as do cultures of *Bacterium granulosis*. The early or acute aspect of human trachoma, illustrated by Noguchi on Plate 1 of his Supplement (2), was used as a standard for comparison with the clinical appearance of the experimental conjunctivitis. Observations of animals with lesions enduring for a period of 1 to over 3 years reveal that the experimentally induced conjunctivitis retains indefinitely its characteristic resemblance ordinarily to the early stage and only occasionally to the beginning of the florid stage of human trachoma. In terms of MacCallan's (7) comprehensive classifi-

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veloped. The eyelid became so edematous and thickened that its eversion for the purpose of examining the conjunctiva was extremely difficult. The skin of the lid sometimes became discolored from subcuticular hemorrhage and localized abscess formation occasionally supervened. After a week the inflammation subsided, and the upper lid and conjunctiva showed, in place of the granular type of reaction, certain marked changes of another kind.

On examining the eyes at this time, a slight greyish yellow discharge was noted, which dried in small flakes on the eyelashes and margins of the lids. At times there was definite ptosis of the upper lid. The upper tarsal conjunctiva was deep red in color, edematous, and thickened, and as a consequence the individual follicles were less prominent than in the granular stage. The edema, thickening, the gelatinous follicles buried in the infiltrated mass of conjunctiva which added a peculiar lustre to the tissue gave rise to a distinct velvety appearance of the tarsal mucosa. Here and there were slight projections of the mucosa which were the result of papillary hypertrophy. The vascular structure, which is seen normally and in certain cases of the granular type of conjunctivitis, was completely obscured. In the upper cul-de-sac, the mucosa was thickened and hyperemic, and the enlarged follicles appeared as slight, rounded elevations, somewhat paler than the surrounding deep red coloration. Expression of these follicles, which appeared like sago grain, yielded a small amount of grumous, greyish, soft material. In the lower lid changes were usually not pronounced: the original granular condition was slightly exaggerated and was accompanied by moderate edema of the tissues. In two of the monkeys, however, changes similar to those occurring in the upper mucosa were noted in the uninoculated eye 2 months after secondary infection was induced, thus indicating a natural extension of this infection. Pannus and lid deformities due to scar tissue formation have not as yet been observed.

In general, the clinical appearance of the animals closely resembled that of Type IIb and c (MacCallan (7)) of human trachoma and agreed with the description given by Morax (10) and others of the florid type of the disease. Two instances of the florid type of the experimental affection are shown in Figs. 1 and 2, and, by way of contrast, a drawing of characteristic, uncomplicated, granular conjunctivitis induced by *Bacterium granulosis* is reproduced in Fig 3.

Various bacteria have been mentioned (7) as occurring during the florid stage of trachoma in man—bacteria which may be regarded as secondary, in view of the fact that Noguchi (2) and ourselves have found them to be devoid of pathogenicity for monkeys and to be indiscriminately associated with the pathogenic *Bacterium granulosis*. As yet, however, no definite evidence has been obtained as to their significance in human trachoma and in the experimental disease.

Materials and Methods

Test Animals.—There were available nine *Macacus rhesus* monkeys showing lesions resembling Type I or Type IIa human trachoma as a result in two instances of subconjunctival inoculation with human trachomatous tissue, and in the other seven of inoculation with cultures of *Bacterium granulosis*. In all, the disease had been progressing over a period of from 2 to 7 months before secondary organisms were implanted experimentally upon the diseased conjunctivae. In addition, five normal monkeys with clear conjunctivae were used as controls for determining the effects of inoculation of the secondary bacteria alone.

Microorganisms for Secondary Inoculation.—The bacteria selected to produce secondary infection consisted of some of those usually encountered in the cultivation of conjunctival tissue from trachoma or other conjunctival affections of man and monkey. They were a Gram-positive diphtheroid, *Staphylococcus albus*, *Bacillus xerosis*, a chromogenic and a spore-bearing bacillus, both Gram-negative, and a non-hemolytic streptococcus.

*Method of Inoculation.*²—The growths were suspended in the condensation water of the tubes, and the turbid fluids were removed by means of pipettes and pooled. To the mixture an equal volume of 0.9 per cent salt solution was added. The application of the culture mixtures to the conjunctivae by daily swabbing over long periods yielded no results in either the monkeys with *granulosis* lesions or the controls, and even the subconjunctival inoculation of 0.2 cc. of the mixtures failed, in most instances, to induce infection.

We then turned to the use of testicular extract (8) for the purpose of enhancing the pathogenic action of the bacteria (9). To suspensions of organisms like those employed previously was added an equal amount of testicular extract (8) and 0.2 cc. of the mixture was forthwith injected subconjunctivally into the animals. The inoculation was repeated twice or thrice at intervals of 3 to 4 weeks. Infection promptly resulted.

Results of Secondary Infection

Clinical Effects.—Immediately after the injection of the mixture of bacteria and testicular extract, a marked, acute conjunctivitis de-

² All operations were done under full ether anesthesia.

The conjunctival tissue of six monkeys with the florid type of trachomatous conjunctivitis, after secondary infection, was examined microscopically and the changes, mainly follicular and papillary, were found to be strikingly similar to those occurring in florid, Type IIb and c human trachoma (Figs. 5 and 6).

In certain areas, particularly in the cul-de-sac, the epithelium was thickened, the cells were swollen and pale, and huge goblet cells were seen. Infrequent mitotic figures were present. Occasional lymphocytes were buried among the epithelial cells, but in the subepithelial layer there was well marked lymphocytic infiltration. Other areas, especially over the follicles, revealed a thinning or a denudation of the epithelium. Here polymorphonuclear leucocytes invaded the remains of necrotic epithelial cells, and the subepithelial structure. Microorganisms of different species could be seen, singly or in an agglomeration, intra- and extracellularly. Many lymphocytes and a few large mononuclear phagocytic cells were scattered about. Numerous connective tissue cells occurred beneath the surface, among which could be found many lymphocytes.

The follicles, similar to those of human trachoma, consisted of large focal collections of tightly packed lymphocytes, some surrounded by a very thin, fibrous tissue capsule. Few mitotic figures were seen, and the number of large mononuclear cells in the center was much less than in the uncomplicated experimental disease. In some of the follicles connective tissue cells could be observed extending inward among the lymphoid cells, while in others there occurred almost complete replacement by scar tissue.

While the deforming effects on the eyelids and their appendages of scar tissue replacement have not as yet been observed in the experimental animals, microscopic examination revealed a considerable degree of fibrosis.

In the control monkeys, those in which secondary microorganisms were injected into the clear conjunctivae, the reaction was one of acute exudative inflammation. The superficial epithelium was denuded, or if attached showed degeneration of the nucleus and cytoplasm to the degree of complete necrosis. In the subepithelial tissue, an exudation of cells, chiefly polymorphonuclear leucocytes and a few large mononuclear cells, occurred. The capillaries were distended, and here and there a small hemorrhage could be seen. Many of the polymorphonuclear and large mononuclear cells contained bacteria. There was no lymphocytic reaction and no scar tissue or follicle formation. The lesion was therefore distinct from that found in the test monkeys (Fig. 7).

The evidence is clear that the predominant reactions in the experimental disease, like those in human trachoma, are lymphoid and papillary hyperplasias associated with processes of definite scar tissue replacement. The pathological changes may therefore be considered

The condition produced by implantation of secondary infection upon experimental granular conjunctivitis continued with only slight remissions from 3 to 7 months, when it was terminated in three monkeys by removal of tissue for study by means of tarsectomy. Four other animals died at periods from 3 to 7 months from tuberculosis and two are still under observation.

In control monkeys the injection of the bacterial mixture was followed by an acute inflammatory reaction consisting of edema and thickening of the eyelids, with an occasional subcuticular hemorrhage and localized abscess formation. The inflammation subsided within a week, leaving the upper and lower conjunctivae somewhat thickener by edema and infiltration. The vascular structure of the mucosa then appeared sharply defined and apart from slight roughness of its surface no papillary or follicular hypertrophy was noticeable (Fig. 4).

Bacteriological Findings.—At the termination of the observations, cultivation tests were made with the conjunctival tissue removed from six of the nine test animals and from one of the controls. With the exception of the Gram-negative spore-bearing bacillus and the streptococcus, bacteria similar to the secondary microorganisms which had been inoculated were recovered from the monkeys. Three of the nine animals yielded *Bacterium granulosis* in culture—a result which was not surprising in view of the fact that the lesions induced by the original inoculation of *Bacterium granulosis* had been present for several months before the secondary infection. One of three monkeys yielding the microorganism had shown lesions for 14 months and two for 11 months. That *Bacterium granulosis* can be recovered from chronic experimental conjunctivitis of long standing (3 years) has already been demonstrated by Tilden and Tyler (4, 11), a finding correlated with the presence of the bacterium in chronic human cases of trachoma of several years' duration (2, 6, 11, 12, 13).

Pathological Changes.—The histopathology of chronic, uncomplicated, granular conjunctivitis induced in monkeys by inoculation of *Bacterium granulosis* has already been described by Noguchi (2). Its resemblance to the microscopic changes resulting from injection of human trachomatous tissues in monkeys has recently been reported (12).

bacteria on the conjunctivae of monkeys already having well marked characteristic *granulosis* lesions might give rise to a condition showing less predominance of the follicular reaction and more of the hyperemic granulopapillary effect. We believe we have succeeded, by a suitable technique, in doing this and in producing thereby in the experimental animals a condition closely resembling the florid type of human trachoma (Type IIb and c of MacCallan). The organisms used were some of those which are found in the conjunctival sac of monkey and man with different types of granular conjunctivitis. Of themselves, they produced only a transient reaction or none, as Noguchi had already shown and as we ourselves had observed in control animals. When inoculated together with material containing *Bacterium granulosis*, they did not affect the usual action of this organism, but when introduced into a conjunctiva in which *granulosis* lesions were already well developed, they induced more of the characteristics of the florid stage of trachoma than have been hitherto observed in animals, notably increased hyperemia, edema and thickening, papillary hypertrophy, obscurity of blood vessels, and masking of the follicles. Microscopically these changes were accompanied by increased scar tissue formation, lymphoid infiltration, and papillary hyperplasia.

CONCLUSIONS

By introducing secondary infections in monkeys already showing characteristic granular conjunctivitis following inoculation of human trachomatous tissues or cultures of *Bacterium granulosis*, it is possible to bring about a condition in which the clinical appearance closely resembles that of florid human trachoma.

Secondary infection appears to be important in the pathogenesis of the experimental disease, since by it a reaction which is mainly follicular can be converted into a still more severe and destructive hyperemic, granulopapillary type. In this respect an analogue may be found to trachoma in man.

We wish to express our gratitude to Dr. Martin Cohen, without whose active cooperation these studies could not have been made.

as those of the florid or Type IIb and c trachoma of MacCallan's terminology.³

In another series of tests a culture of *Bacterium granulosis* was inoculated into the smooth conjunctivae of two monkeys, and the same culture plus an equal amount of the testicular extract suspension of the secondary microorganisms into two other animals. Two additional monkeys served as controls to the action of the secondary bacteria alone. The latter animals revealed the acute inflammatory reaction already described. All four monkeys receiving *Bacterium granulosis* with or without secondary microorganisms developed the conjunctivitis characteristic of *granulosis* alone, without any distinctive differences in the two pairs of animals.

It appears from the foregoing experiment that the florid type of trachoma can be produced experimentally by secondarily infecting conjunctivae which show follicular lesions. The simple mixture of *Bacterium granulosis* and other microorganisms may induce only the effects of the pathogenic constituent, namely, the *granulosis* organism, and the process is comparable to the action in the conjunctiva of affected tissues from man or monkey, in which there is usually an admixture of the pathogenic microorganism with different bacteria. The procedure can be likened to that of injecting pneumonia (pneumococcus) sputum into the susceptible mouse.

SUMMARY

The possibility suggested itself, in view of the theories already advanced with regard to the rôle of secondary infections in the later manifestations of trachoma (1), that the implantation of common

³ It has been implied recently (14) that atropine can induce follicular conjunctivitis and that this condition should not be confused with trachoma. We have injected subconjunctivally two monkeys with 0.2 cc. of 0.5 per cent solution of atropine sulfate. Both monkeys showed, after 24 hours, considerable edema of the eyelids and acute hemorrhagic conjunctivitis. After 5 days the inflammation subsided, except for the persistence of retrolarsal hemorrhages, and after 3 weeks the eyes were practically normal. A week later both monkeys were reinoculated subconjunctivally, this time with a *Bacterium granulosis* culture. From 20 to 30 days thereafter, they developed characteristic experimental granular conjunctivitis. The atropine effect was therefore wholly unlike the experimental disease induced by the microorganism.

PLATE 26

FIG. 5. Histopathological changes in conjunctiva of a monkey with secondary infection of chronic granular conjunctivitis. To be noted are two follicles and the changes described in the text. Duration of secondary infection 5 months. Hematoxylin-eosin stain. $\times 125$.

FIG. 6. Similarly, from a case of florid human trachoma, of about 2 years' duration. Same stain. $\times 60$. To be compared with Fig. 5.

FIG. 7. Conjunctiva of a control monkey similar to that shown in Fig. 4. There was no evidence of follicle formation. Conjunctiva removed 7 months after the initial, and 34 days after the last injection of secondary microorganisms. Same stain. $\times 500$.

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EXPLANATION OF PLATES

PLATE 25

FIGS. 1 to 4. Conjunctivae of *Macacus rhesus* monkeys enlarged to twice natural size.

FIG. 1. Secondary infection of chronic granular conjunctivitis following subconjunctival inoculation (January 9, 1930) of a culture of *Bacterium granulosus* derived from a case of trachoma in New York City. Duration of secondary infection 5 months.

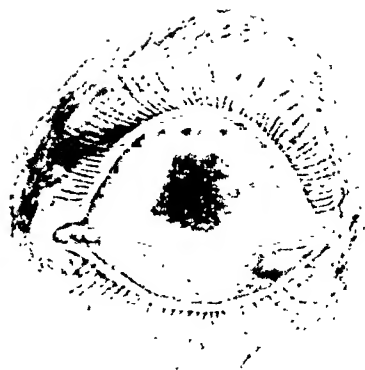
FIG. 2. Conjunctiva of another monkey treated in the same way. The sago-like follicles mentioned in the text should be noted. Duration of secondary infection 6 months.

FIG. 3. Uncomplicated chronic granular conjunctivitis resulting from subconjunctival inoculation of *Bacterium granulosus* derived from a case of human trachoma. The drawing was made 65 days after inoculation. To be compared with Figs. 1 and 2 which show the results of secondary infection produced in monkeys having originally lesions similar to those of Fig. 3.

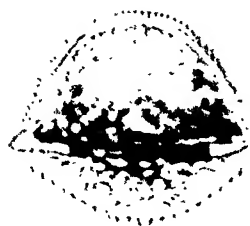
FIG. 4. The effect of subconjunctival inoculation of the ordinary bacteria found in the conjunctiva of man and the monkey. Control animal to those of the series represented in Figs. 1 and 2.



1



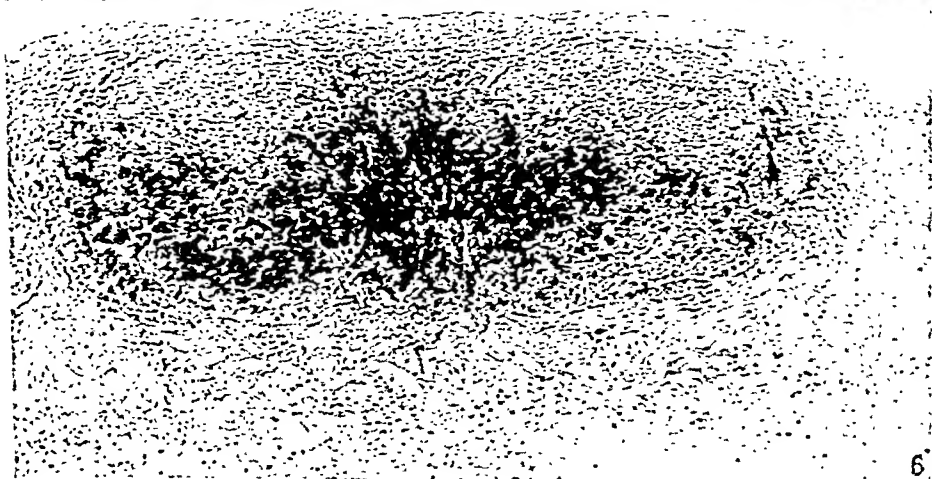
2



3



4



these complex mechanisms and the examination of them under conditions of isolation and control.

The method of perfusion of the frog's kidney approaches at least the accomplishment of this ideal. Its arterial-glomerular and venous-tubular circulation, together with the possibility of administering dyes to it which are reciprocally excreted in significant amount by only one of the renal mechanisms, is the basis of its value. Controversy concerning these concepts indeed exists, but as will be shown later, the use of the method with the pathological kidney adds further evidence of their verity, in the same way that the use of toxic damage by Bainbridge, Collins, and Menzies (3) and Höber (4) has illuminated obscure aspects of normal kidney function.

Technique

The method of perfusion has been previously described (5). It is based on Höber's (4) modification of the Barkan, Broemser, and Hahn (6) technique by which isotonic Locke's solution containing 0.025 per cent sugar and a small amount of glycol maintained at a pH of 7.3 is led from separate containers to the arterial and the portal-renal venous systems. The urine is collected from each kidney in cannulae. The urine formed by the procedure when successful is sugar-free, its electrolyte content is less than one-half that of the perfusion fluid, and the rate of its excretion is comparable to that of the formation of urine by the living frog. If urea is added to the perfusion fluid it is concentrated in the urine. Failure in method is therefore easily detected by the presence of sugar in the urine, by a rise in salt content towards the level existing in the perfusion fluid, or by abnormal rates, either high or low, of water excretion. The methods of determination of the constituents of the urine were as follows: Benedict's method for sugar, determination of electrolyte content by the Christiansen ionometer, and dye content by the usual colorimetric method.

The Manner of Excretion of Phenol Red and Neutral Red

We have previously stated our reasons for believing that phenol red is excreted by the perfused frog's kidney chiefly through the glomerulus and that neutral red is excreted principally through the tubular epithelium. Misunderstanding may perhaps be avoided if we emphasize that our interest in the present study is not how these dyes are eliminated by the kidney of the living frog but how they are excreted by the isolated kidney under our experimental procedure, for these procedures as used here are not designed to examine the intricacies of

EXPERIMENTAL NEPHRITIS IN THE FROG

II. PERFUSION METHODS OF TESTING THE KIDNEY BY DISSOCIATION OF ITS FUNCTIONS*

BY JEAN OLIVER, M.D., AND ESHREF SHEVKY, Ph.D.

(From the Departments of Pathology of the Long Island College of Medicine, Brooklyn, and of Stanford University Medical School, San Francisco)

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The hypothesis that correlation of function and anatomical change may be more accurately examined by the study of experimental nephritis in the kidney of the frog than in the complex mammalian kidney is the basis of the present work. A preliminary investigation (1) has shown that the morphological changes observed in mammals after the kidney is damaged by renal toxic agents are reproduced in all their essential characteristics in these relatively simple kidneys when such substances are administered *in vivo* to the frog. Moreover it has been found that in the frog the consequent lesions are less complex and are therefore more readily open to interpretation. With this encouragement the next step has been an examination of function.

As we have pointed out in another place (2), the chief factor that makes it difficult to achieve any successful result with the mammalian kidney in such a correlation, is the impossibility of distinguishing in the total function of the kidney the function of its component parts. For example, judging from present physiological experience, damage to tubules may compensate for disturbances due to damage in the glomeruli and the consequent presence of two or perhaps more variables leaves the observer confused. In studying the problem in the frog our attention must therefore be directed towards a dissociation of

* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacology and Chemistry, American Medical Association.

further these values reached ultimately an amount approximately one-tenth of their original normal values, the urine was highly concentrated, and sugar disappeared from it.

At Period 12 the arterial supply was reestablished. The volume of urine thereupon increased to its original value, and was accompanied by increases in the rate of phenol red and salt excretion which also approached their normal figures. The final conditions, including the absence of sugar from the urine, therefore approximated the original conditions of the experiment.

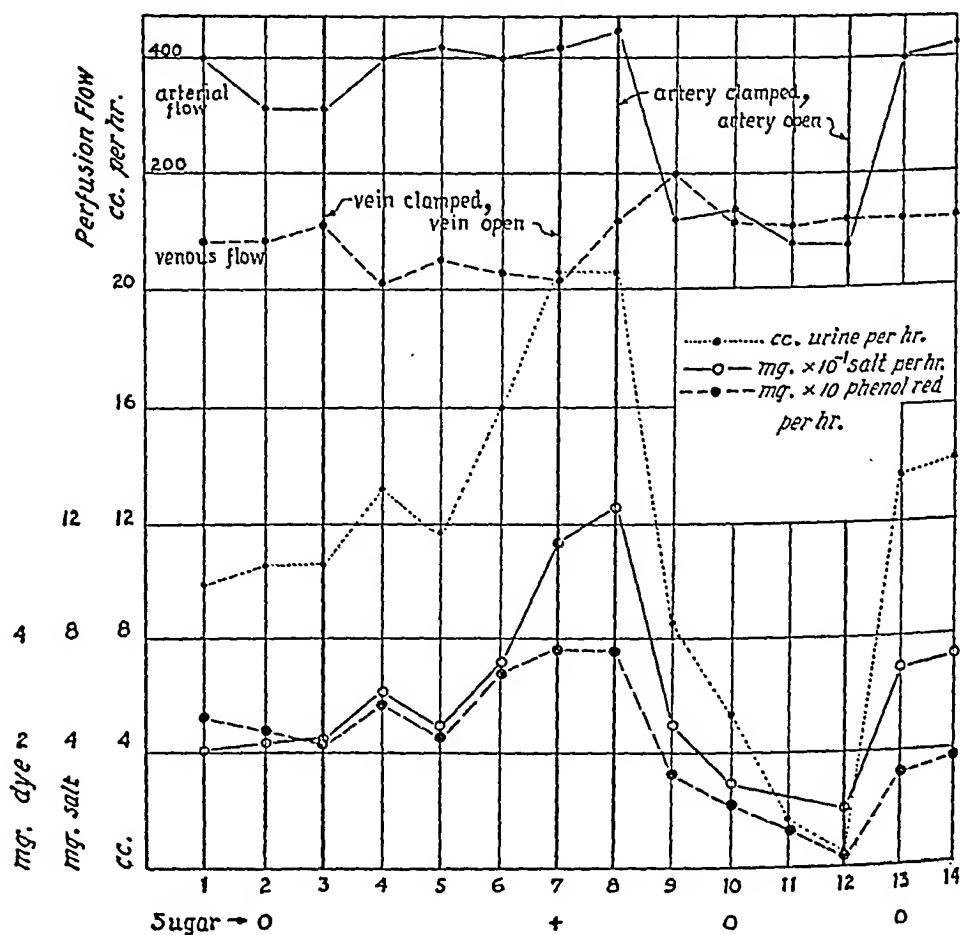


CHART 1

The experiment emphasizes certain points. First, that an increase in urine volume may result from a *restriction* of the supply of fluid to the tubules but that the ultimate source of water depends on adequate glomerular supply. Second, that a restriction of the supply of fluid to the tubules produces those changes which have formerly been re-

renal physiology, but to test the reaction of the two components of the renal mechanism under abnormal conditions.

Dissociation of Glomerular and Tubular Function

In the study of the function of the normal kidney a frequent procedure is to damage or narcotize one or the other of the kidney elements on the assumption that this depresses its activity and hence removes its influence from the total function of the kidney. This method has been applied especially to the examination of tubular activity. An excessive excretion of water or the appearance of sugar in the urine after such treatment of the tubules has therefore been interpreted as the result of an abolition of a normal absorptive function inherent in them. Although there is a considerable amount of indirect evidence to support this idea, the fact remains that there are other possible interpretations. The concept of tubular absorption depends largely on the assumption that the bulk of the water in the urine is derived from the glomeruli. Moreover it is entirely possible that damage to the tubular epithelium might allow both water and sugar to leak through its wall from the surrounding capillaries and thus explain the experimental findings. Further evidence on the question is therefore needed and the following experiment, an example from a series of twelve essentially similar findings, is given.

The isolated kidneys of *R. catesbiana* were perfused through both the arteries and the renal-portal vein in the usual way with modified Locke's solution containing 0.05 per cent phenol red and 0.025 per cent sugar (Chart 1). As soon as a normal output of urine was established the flow through the renal-portal venous system was gradually restricted until in the fourth, fifth, and sixth periods of the experiment it was only a fraction of its original figure. The tubules were thus deprived of a part of their supply of fluid. It will be seen that there resulted a gradually increasing output in the amount of water until in the seventh period a rate of 20.8 cc. per hour was reached, more than twice the original volume. Accompanying this diuresis there went an increase in the rate of salt and phenol red excretion and sugar appeared in the urine.

In the seventh period the restriction of the venous-tubular supply was removed. The supply of fluid to the tubules was thus increased and in the next (eighth) period the flow of fluid through the arterial system was reduced. A sharp fall in water output immediately resulted and with this fall a decrease in the output of salt and phenol red occurred so that the original rates of excretion of these two substances were again reached. As the supply of arterial fluid was decreased still

stances an attempt at simplification and control has been made in that we have not given the poison to the living animal where its activity may be modified by the infinite complexities that of necessity develop when a substance is introduced into the living organism, but to the organ itself isolated from these uncontrollable and in great part unknown factors. Furthermore, in the frog's kidney at least, such a method allows to a certain degree the administration of the toxic agent to specific structures in the organ, and the concentration of its effects to definite localities. The importance of this factor is apparent when one calls to mind the current theory which claims that renal toxins are by themselves able to produce specific lesions in one or the other part of the renal unit.

Even under these controlled conditions and with the relatively simple kidney of the frog, as the experiments will show the reactions are exceedingly complex. We can only describe, therefore, the simpler and more typical of the "syndromes" which are observed. No attempt has been made to determine the action of all the known renal poisons as we are interested at this time only in methods which allow us to recognize where the damage to the kidney has been done. A study of the action of the poison itself will be considered at a later time when the kidney of the nephritic frog is examined.

Evidences of Absorptive Failure

If a renal toxic agent is introduced into the renal-portal circulation of the perfused kidney, the arterial circulation remaining normal, a damage to the tubular elements occurs whose functional aspects correspond exactly with those phenomena which our basic experiment has shown to be the result of a depression of the absorptive function of the tubule. Sugar appears in the urine, there is an increased output of water, and an increased excretion of salts. The rate of excretion of phenol red is not significantly decreased and may in fact be increased. Experiments illustrating these effects as a result of several toxic agents are given in Table I.

In these experiments it will be noted that no significant alteration in the flow of the perfusion fluid followed the administration of the toxic substance. Attention should be drawn to the fact that the lack of function of the tubular epithelium following urethane was not

ported as a result of damage by toxic agents. The experiment therefore supports the theory that diuresis, increased salt output, and appearance of sugar in the urine are not the result of any increased passage of these substances through a damaged tubular epithelium but that they are due rather to a lack of absorptive activity of this portion of kidney mechanism. In other words, these phenomena represent an abolition of a normal absorptive function, a disturbance which in our experiment was due to a lack of an essential supply of materials to the tubule cells.

It would also appear that the source of the greater part of phenol red in the urine is the glomerular apparatus, since the proper excretion of this substance in our experiment is dependent on a proper supply of fluid and dye to this structure. This fact and its converse, that neutral red is excreted in greater part through the tubular epithelium, we shall not discuss at this time. Experiments similar to the one we have just described may be found in another place (7) where it has been shown that though the changes in water, salt, and sugar excretion are consistently similar no matter which dye is used in the perfusion fluid, the dyes themselves behave in an opposite manner to the experimental variations in fluid supply.

These experiments in which it is believed glomerular and tubular functions have been dissociated to such an extent that their individual characteristics may be recognized, form the basis for the interpretation of the results in the varied experiments that follow. Certain phenomena such as diuresis, increase in salt output, the appearance of sugar in the urine, or fall in rate of phenol red or of neutral red elimination, to name only a few, have been found to follow mechanical and relatively simple procedures, such as reducing the supply of fluid, or its pressure, to a certain part of the kidney unit. It is by the recognition of these same functional variations in experiments where toxic substances have damaged the kidney that the attempt to localize the seat of lesion will be made.

The Demonstration of Tubular Dysfunction by the Administration of Toxic Agents to the Kidneys

In devising methods for the detection of those disturbances of function in the kidney that follow the administration of toxic sub-

tivity may be accompanied by a decrease or even complete failure of the secretory activity of the tubule cells. In the experiments of Table II neutral red was added in concentrations varying from 0.01 to 0.02 per cent to the perfusion fluid which was passing by way of the renal-portal vein to the tubules. In the first two experiments the toxic agent was introduced into the venous circulation after the establishment of normal urine formation and when a normal excretion

TABLE II
Evidences of Secretory Failure

	Arterial flow	Venous flow	Urine flow	Neutral red	Salt	Sugar
Urethane						
	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>	
11:30-11:40	540	600	7.8	0.63	42.9	0
11:40	2 per cent urethane to tubules throughout following periods					
11:40-11:50	600	540	18.0	0.36	99.0	+
11:50-12:00	600	600	21.0	0.36	115.0	++
12:00-12:10	540	660	21.6	0.26	147.0	++
Potassium bichromate						
10:45-11:00	480	660	3.6	2.01	—	0
11:02-11:05	10 cc. 1/2000 potassium bichromate to tubules					
11:15-11:30	320	320	4.0	1.12	—	+
11:30-11:45	300	520	4.6	1.09	—	++
Corrosive sublimate						
11:45-12:00	360	440	4.8	—	24.0	0
12:00-12:03	10 cc. of 1/2500 sublimate to tubules					
	Neutral red to venous fluid supply					
12:15-12:30	200	480	8.0	Ftst. tr.	48.0	+
1:30- 1:45	160	480	1.2	Ftst. tr.	9.0	++

of the dye was in process. Along with the evidences of absorptive failure, diuresis, high salt output, and glycosuria, there went a definite decrease in the rate of excretion of neutral red. In the third experiment, after normal urine formation was established by the perfusion with clear Locke's solution, the tubule cells were damaged by the toxic agent. After a proper interval, which was characterized by the same absorptive abnormalities described above, neutral red

simple anesthesia for there was no recovery of tubular activity when the urethane was discontinued, and finally, that we are warranted in assuming that actual tissue damage occurred after the introduction of all these substances, will be apparent in a later description of the anatomical changes that occurred in the kidneys of these and similar experiments.

TABLE I
Evidences of Absorptive Failure

	Arterial flow	Venous flow	Urine flow	Phenol red	Salt	Sugar
Corrosive sublimate						
11:40-11:50	cc. per hr. 540	cc. per hr. 500	cc. per hr. 7.8	mg. per hr. 1.01	mg. per hr. 42.9	0
11:50-11:53		10 cc. 1/10,000 sublimate to tubules				
12:00-12:10	600	180	13.2	1.25	72.6	+
12:10-12:20	540	200	12.0	0.78	72.0	++
Uranium nitrate						
11:30-11:45	240	280	2.4	1.09	—	0
11:45-11:50		15 cc. 1/5000 uranium nitrate to tubules				
12:00-12:15	120	280	2.4	—	—	0
12:15-12:30	280	400	6.4	0.740	—	Tr.
12:30-12:45	120	400	5.2	0.806	—	+
Urethane						
11:20-11:30	540	300	12.0	1.2	66.0	0
11:30		2 per cent urethane to tubules continuously through following periods				
11:30-11:45	540	540	17.4	1.4	104.0	0
11:45-12:00	600	480	20.0	1.0	110.0	Tr.
12:00-12:15	600	520	25.4	1.0	152.0	+

*Evidences of Secretory Failure**

When the perfused kidney is treated as described above it can be shown by testing with neutral red that the failure of absorptive ac-

* The term secretion is used here in only a general sense to denote the passage of a substance from the blood stream through the epithelial cell into the lumen of the tubule. The term excretion is applied to the activity of the kidney as a whole. As will be seen the *excretory* activity of the kidney may be increased by changes which at the same time abolish the *secretory* activity of the tubular epithelium.

stance is introduced into the arteries and thus reaches the glomeruli in high concentration, a trace of precipitate increasing to a light or

TABLE III
Dissociation of the Tubular Functions

	Arterial flow	Venous flow	Urine flow	Neutral red	Salt	Sugar
Potassium bichromate						
Secretion of neutral red and absorption of water, normal: absorption of sugar, abnormal						
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	mg. per hr.	
10:30-10:45	360	630	4.0	0.52	—	0
10:45-10:48	15 cc. 1/3000 potassium bichromate to tubules					
10:50-11:05	440	100	4.0	0.60	—	Tr.
11:05-11:20	400	560	4.4	0.62	—	+
Potassium bichromate						
Secretion of neutral red, normal: absorption of water and sugar impaired						
10:30-10:45	600	600	4.4	7.7	—	0
10:45-10:48	15 cc. 1/4000 potassium bichromate to tubules					
11:00-11:45	600	640	7.2	7.9	—	0
11:15-11:30	520	500	6.0	6.0	—	+
Urethane						
Permanent failure of neutral red secretion: transient failure of water and sugar absorption with recovery						
10:45-11:00	320	600	3.2	2.7	—	0
11:00	0.5 per cent urethane to tubules through following periods					
11:00-11:15	320	280	2.4	0.40	—	+
11:15-11:30	320	440	8.0	1.7	—	+
11:30	Urethane to tubules stopped					
11:20-11:45	320	380	6.4	1.6	—	+
12:00-12:15	240	240	4.0	0.8	—	Tr.
12:30-12:45	280	240	3.2	0.5	—	Ft. tr.
Phloridzin						
Failure of neutral red secretion: failure of sugar absorption, maintenance of water and salt absorption						
11:00-11:15	360	440	4.4	8.8	15.4	0
11:15-11:18	15 cc. 1/15,000 phloridzin to tubule					
11:30-11:45	360	280	3.2	1.2	11.2	+
11:45-12:00	280	360	4.4	1.1	17.6	+

heavy cloud promptly develops. Table IV shows the results of an experiment where paraphenyldiamine was administered first to the

was then supplied to the tubules. It will be seen that there was an almost complete lack of elimination of the dye in spite of the relatively large output of water, salt, and sugar, conditions which, as other experiments have shown, are not incompatible with the excretion of considerable amounts of phenol red.

The Dissociation of the Tubular Functions

The question arises if it is possible to produce one of the phases of tubular functional damage without the appearance of the other. Both depression of absorptive activity with intact secretory function and the converse condition of depressed secretory function and normal absorption by the tubule cells have been observed. The former case, however, has been less frequently encountered than the latter. Table III gives examples of such experiments.

The Demonstration of Glomerular Dysfunction

(a) Evidences of Increased Filtration

Our experiments have given no direct evidence as to whether the mechanism of passage of substances through the glomerular membrane is one of filtration or active secretion. Under certain pathological conditions at least the process closely resembles filtration and we have therefore used this term descriptively with reservations as to the exact nature of the process.

The simplest and most objective demonstration of an increase in the permeability of the glomerular membrane is obtained by testing it with substances which do not pass through it and appear in the urine under normal conditions. One thinks immediately of the proteins of the blood plasma which are thus held back by the normal kidney and in analogy to them the hydrophilic colloid gum arabic was used. If this substance is added in a concentration of 1.0 per cent to the perfusion fluid and passed through both the arterial and venous circulations of the frog's kidney, it does not appear in the urine so long as the urine is normal. If the tubules are damaged by a toxic substance and the typical results of such damage as described above appear, the urine may still remain free of gum when tested with a concentrated solution of picric acid. If, however, the toxic sub-

as those which demonstrated its increased permeability for other constituents of the urine.

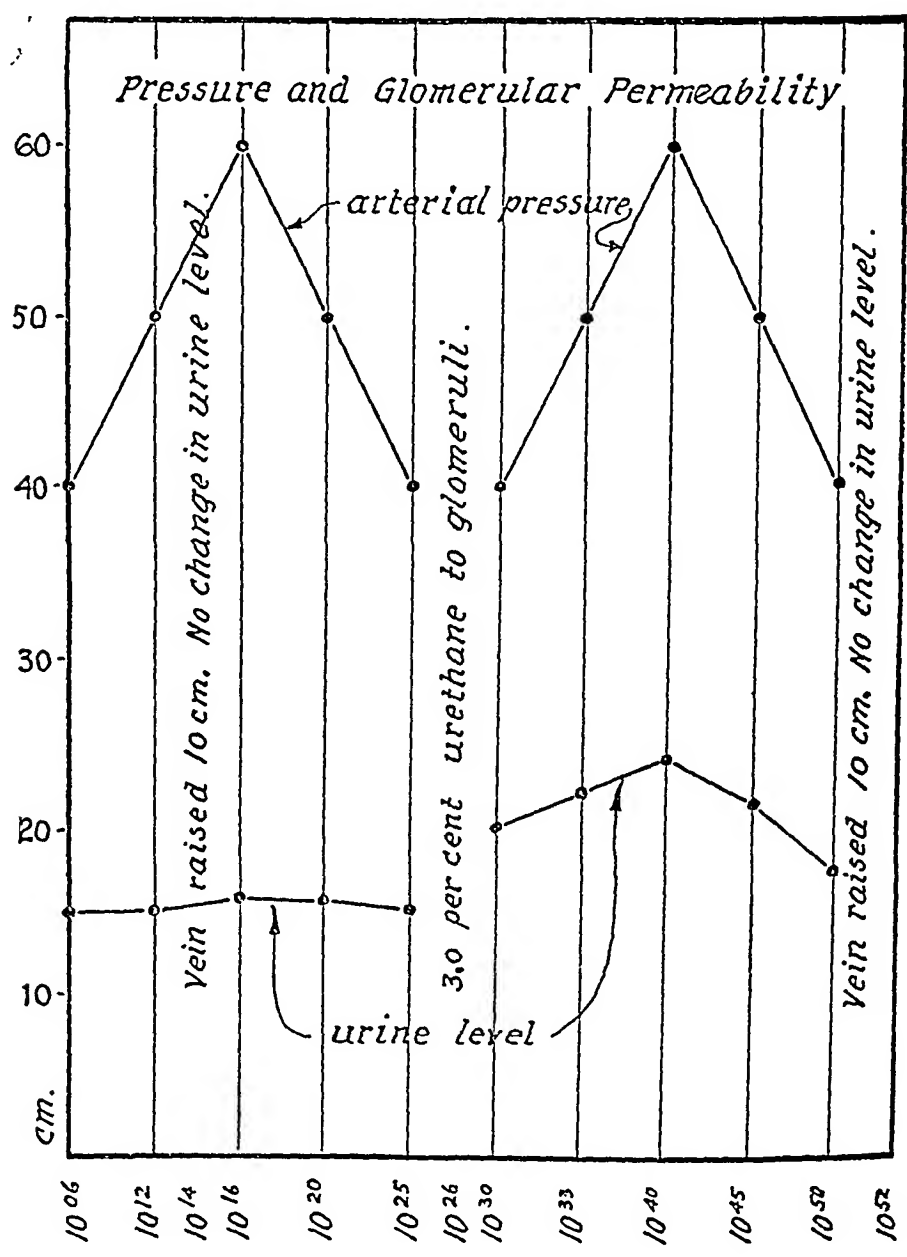


CHART 2

If a tube is placed in the ureter of one kidney and so fixed that it stands perpendicular the urine as it is formed by the perfusion will

tubules and then to the glomeruli by way of the arteries after the perfused kidney had established an output of normal urine.

As we have shown in another place the excretion of such colloidal dyes as brilliant red and trypan blue is also altered by damage to the glomerular membrane (8). The former does not pass through the normal membrane, but appears in the urine if this structure is damaged, while the latter alters the color of the urine, in which it normally appears in small amount, not only quantitatively but also qualitatively as the damage and consequent permeability of the membrane increases.

TABLE IV
Evidences of Increased Filtration

	Arterial flow	Venous flow	Urine volume	Phenol red	Salt	Sugar	Gum
Paraphenyldiamine							
	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>mg. per hr.</i>	<i>mg. per hr.</i>		
11:15-11:30	300	480	4.2	0.31	21.0	0	0
11:30-11:35	10 cc. 0.8 per cent paraphenyldiamine to tubules						
11:45-12:00	360	300	4.5	0.27	27.0	0	0
12:00-12:15	360	300	6.3	0.36	37.0	0	0
12:15-12:18	10 cc. 0.8 per cent paraphenyldiamine to glomeruli						
12:30-12:45	150	240	3.6	0.29	21.6	0	Tr.
12:15-1:00	220	480	4.2	0.22	25.2	0	++

It would seem likely that if the glomerular membrane becomes more permeable to substances in the perfusion fluid it should under these conditions allow an increased passage of water. A direct determination of this point is however difficult, for a similar end result, that is an increase in the volume of the urine, is obtained when the tubular absorptive activity is depressed. As we shall show later a combined damage to both parts of the renal unit sometimes follows the introduction of the toxic agent into either one of the renal circulations, and special means not always possible in an experiment designed for some specific purpose may be necessary to discover that more than one element of the kidney is affected. The experiment we shall describe is therefore not as direct a demonstration of increased permeability to water following membrane damage

(b) Evidence of Decreased Filtration

Decreased filtration may be the result of at least two mechanisms. The amount of fluid available for filtration may be decreased and, by what amounts to the same thing, there may be a decrease in the number of filters in action. A decrease in the pressure head of the fluid may in a similar way lead to the same result. Or the membrane of the filters may become more dense and even with pressure or amount of fluid remaining constant there will result a decrease in the amount of filtrate.

The first mechanism is definitely demonstrated in our basic experiment as a result of restriction of arterial supply. Doubtless both fall of pressure and decrease in fluid were factors in its production, and the same mechanism, a result of arterial spasm, may be observed when toxic agents are introduced into the arterial circulation. Table V illustrates such an experiment. The perfused kidneys were functioning normally and flow through the arterial and venous circulations was moderate yet adequate and constant from period to period of the experiment. Phenol red was excreted in moderate amount. 15 cc. of 1/7500 sublimate were slowly introduced through the arterial circulation to the glomeruli. There resulted a gradual drop in the flow through the arterial circulation, from 440 cc. per hour to 140 cc. per hour. Concomitantly there occurred a marked fall in the volume of urine and a consequent decrease in the rate of excretion of phenol red and salts. This decrease in urine continued until in the fifth period anuria was approached.

Though it has no bearing on the question of glomerular dysfunction we mention in passing the effects of spasm in the venous circulation of the perfused kidney. Its result may be predicted from the findings of our basic experiment in which the restriction of the venous circulation was a simple mechanical one. The contrast of the effects of spasm in the venous system that may follow the introduction of a drug into its circulation with the results of the analogous condition in the arterial system is shown in the second experiment of Table V. The perfused kidney was excreting a normal amount of normal urine. 10 cc. of 1/4000 potassium bichromate was introduced into the vein. There resulted a marked reduction in flow through the venous sys-

rise to a certain height and then remain stationary. That this height depends in part at least on the height of the pressure head of the arterial perfusion fluid is evident from the fact that if the level of the arterial perfusion column is further raised, there is an accompanying rise in the level of the urine in the tube. A point is reached, however, when further elevations of the perfusion pressure, if moderate, produce little effect on the height of the urine. If the pressure is raised beyond this point to an extreme degree rupture of the vessels results and perfusion fluid as such flows through the kidney tubules into the upright tube. The following experiment shows the use of these points in our problem.

After normal urine formation had been established by the perfusion a glass tube was placed as described above in the ureter of the right kidney. The height of the arterial perfusion bottle was 40 cm. The urine rose slowly in the tube until it reached a height of 15 cm. and then remained stationary. The arterial bottle was then raised 10 cm. to a height of 50 cm. No change occurred in the level of the urine. Raising the level of the venous supply 10 cm. also produced no change. The arterial pressure was now raised to 60 cm. and a rise of 7 mm. in the urine was noted. It was then lowered to 50 cm., the urine falling 2 mm., and finally it was replaced at its original height of 40 cm., the urine level being now 15.2 cm.

3 per cent urethane was now added to the perfusion fluid which was passing through the arterial circulation. The catheter tube was emptied and allowed to fill again. The urine, with the arterial pressure level at 40 cm. now rose to 20 cm. when the pressure was increased to 50 cm. the urine rose 20 mm., and when raised to 60 cm. the urine level was increased 40 mm. over its original figure. As the arterial bottle was lowered to 50 and then to 40 cm., the urine level fell 25 and 60 mm. from its maximum height. At this point the venous pressure was increased 10 cm. with no effect on the level of the urine.

These results showing the reaction of a damaged glomerular membrane to the passage of fluid as affected by pressure are illustrated in Chart 2.

Such experiments are interpreted to mean that a damaged glomerular membrane allows the passage of fluid more readily when the pressure upon it is changed than does a normal one which offers a certain resistance to moderate variations in pressure. The fact that an increase in the venous pressure produces no change in the amount of urine secreted also adds evidence to the theory that water under abnormal conditions does not enter the lumen of the tubules in significant amount through the damaged tubular wall.

flowed through the arterial circulation only 0.5 cc. per hour of urine was formed. Since by far the greater amount of this perfusion fluid must have passed through the glomerular capillaries the conclusion is possible that fluid did not filter through the glomerular membrane. Another possible explanation will however be mentioned in the discussion.

The Demonstration of Combined Tubular and Glomerular Damage

We have already mentioned the possibility of damage to both elements of the kidney unit even when the toxic agent is introduced with care into only one of the circulations. This is possible not only by communicating channels that exist between the two circulatory

TABLE VI
Decreased Filtration with Adequate Arterial Supply

	Arterial flow	Venous flow	Urine volume	Phenol red	Salt	Sugar
Corrosive sublimate						
1:45-2:00	360	200	6.0	0.12	42.0	0
2:00-2:05	15 cc.1/15,000 sublimate to glomeruli					
2:15-2:30	280	200	4.0	0.08	24.0	+
3:00-3:15	240	280	1.6	0.03	11.0	+
3:45-4:00	340	200	0.5	0.01	3.0	—

beds and by diffusion directly through the tissues but since in most cases the toxic substance is a diffusible one it may pass through the glomerular membrane along with the other constituents of the urine into the tubular lumen and thus come in contact with the tubular cells. As a result we have found it very difficult to damage the glomerular membrane without producing some lesion in the tubular epithelium.

The importance of these facts becomes evident in such an experiment as follows. Sublimate is introduced in low concentration into the arterial system, no arterial spasm results, and there follows a diuresis with increased phenol red and salt excretion. Sugar is present in small amount, perhaps only in traces. The question now arises if all these phenomena may be the result of a marked increase in the

tem, but the volume of urine increased and sugar appeared in the urine. It is of course, impossible in such an experiment to estimate how much of the failure of absorption of water and sugar was due to vascular constriction and how much was the result of damage to the tubule cells from the direct toxic action of the drug.

As previous experiments have shown, glomeruli which have been subjected to the action of such a substance as sublimate become more permeable to the passage of fluid and when examined histologically are found to be definitely damaged. The significance of the coexistence of an increased permeability and decreased volume of urine from vascular spasm will be considered in the discussion.

TABLE V
Evidences of Decreased Filtration

	Arterial flow	Venous flow	Volume urine	Phenol red	Salt	Sugar
	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>cc. per hr.</i>	<i>mg. per hr.</i>		
1:00-1:15	440	120	12.0	0.36	72.0	—
1:15-1:18		15 cc. 1/7500 sublimate to glomeruli				
1:30-1:45	300	120	7.6	0.23	56.0	—
1:45-2:00	300	120	4.8	0.19	33.6	—
2:15-2:30	200	120	0.2	—	1.4	—
2:30-2:45	140	120	0.1	0.003	0.7	—
Effect of venous spasm						
10:40-10:55	400	400	4.8	—	—	0
10:55-11:00		10 cc. 1/4000 potassium bichromate to tubules				
11:00-11:15	240	40	4.8	—	—	Tr.
11:15-11:30	280	80	7.2	—	—	+

The other possible cause of decreased filtration, that is an increase in density of the glomerular membrane which may lessen the filtrate, can be only indirectly and perhaps not definitely demonstrated. In a certain number of cases after the toxic substance has passed to the glomeruli a decrease in the volume of urine occurred sometimes even to the establishment of an anuria without any lessening of flow through the glomerular circulation. Table VI gives an example of such an experiment.

It will be observed that although 340 cc. per hour of perfusion fluid

of the sort that we have been describing. Very commonly vascular spasm complicates the action of either glomerulus or tubule, as for example, when after the introduction of sublimate through the ar-

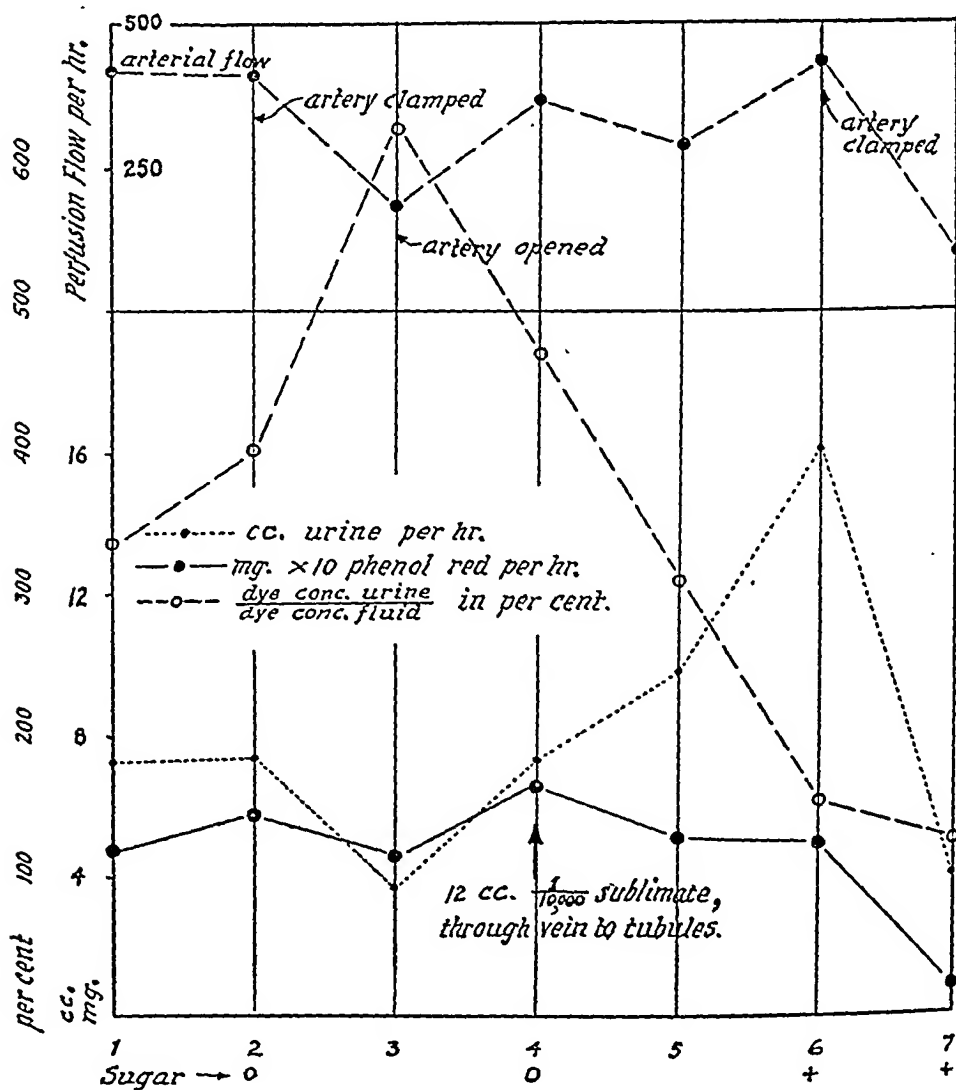


CHART 3

teries, decreased filtration, a result of arterial spasm, prevents a diuresis from the concomitant tubular lesion. Economy of presentation prevents a description of all the reactions we have observed and since in most cases they constitute variations of the simpler ones we

filtrate through the damaged and therefore more permeable membrane, the amount of the filtrate being too great for the absorptive ability of an intact tubular epithelium, or whether the excess water, salt, phenol red, and sugar are in part if not wholly due to an associated tubular damage. Since our ultimate problem will be to test the function of kidneys damaged *in vivo* by agents which have had access to all parts of the renal unit it is obvious that some method must be found which will disclose evidence of tubular damage when it is masked by the results of glomerular lesions.

This may be accomplished if the amount of filtrate is reduced by mechanically restricting the flow through the arterial circulation and observing if tubular absorption is then able with a small amount of filtrate, to concentrate the urine. Chart 3 shows an experiment which illustrates the method.

Normal urine formation was established by the perfused kidney. After the two 15 minute periods the volume was normal, 7.2 cc. per hour, phenol red was excreted at a normal rate and well concentrated and no sugar was present. At the end of the second period the arterial flow was reduced to about one-third its original amount. The volume of urine decreased about one-half and the concentration of phenol red rose, though the rate of elimination was definitely decreased. There was therefore evidence of efficient tubular activity. The reestablishment of the arterial supply increased the rate of phenol red excretion and produced the original conditions of normal concentration and volume. At the end of the fourth period the tubules were damaged by the passage of 12 cc. of 1/10,000 sublimate dissolved in Locke's solution through the venous circulation. During the next two periods there developed typical functional evidence of tubular damage, a diuresis with the appearance of sugar in the urine and though the concentration of phenol red fell sharply, the rate of its elimination remained fairly constant. The arterial supply was now restricted as before, and the volume of urine decreased to one-fourth its former figure. The concentration of phenol red did not rise however as previously noted but actually decreased somewhat.

An interpretation of these results to coincide with the theory stated above is possible. Intact tubules are able to concentrate phenol red more efficiently when the volume of glomerular fluid is lessened, but damaged tubules are unable to raise its concentration even under these optimal conditions.

Beside such combinations of glomerular and tubular damage many other combinations of lesions may be found in a series of experiments

kidney's response. Actually, however, the situation does not prove as hopeless as our analysis would seem to indicate. In the majority of experiments response is determined by the experimental procedure. The toxic agent is confined in its action to the mechanism towards which it was directed and departure from this typical result can in many cases at least be detected, as we have shown, by proper methods of testing. However, our experiments are admittedly incomplete in many regards. For example, the experiment of Table VI, in which a repression of urine was found associated with a good flow through the glomerular capillaries, was interpreted to demonstrate the possibility of an increase in the density of the glomerular membrane as a cause of a lessened filtration of water. Richards (9) by direct visual observation of the kidney of the living frog after poisoning with sublimate has observed that the glomerular circulation may be active although no urine is being formed. The anuria he explains as due to an increased absorption of water by the damaged and therefore more permeable epithelium. Our experiments with a damaged tubular epithelium, and those of the following paper which show that this damage is a complete and structural one, have, in the absence of complicating vascular spasm, been accompanied by failure of rather than by increase of absorption, so that we have preferred the explanation we have given. This is only one of the many points that must be further examined, and it is therefore not so much the detailed findings of our experiments that we would emphasize as the importance of their method.

We believe it would be premature to draw any conclusions as yet in regard to the theory of disturbed kidney function even in the circumscribed field of experimental nephritis. This may be left until we have examined the function of kidneys from nephritic frogs. This has been done by the methods we have described in this study and will be reported later. Certain points deserve emphasis however and may be mentioned now, for they bear on general problems of interpretation of histological appearance in abnormal kidneys no matter what the cause of the abnormality may be. The literature of Bright's disease is full of assumptions as to the effect of damage to one part of the kidney or the other that have had hitherto neither support nor criticism except such as was logically convenient to the development of theory.

have described and may be recognized as such, a brief consideration of them is reserved for the discussion of our experimental findings.

DISCUSSION

Although perhaps no demonstration of the complexity of the reaction of the kidney to the introduction of a toxic agent is needed, the experiments we have described have at least the virtue of pointing out a few definite reasons for some of the complications. Any analysis, even if imperfect, is to be preferred to the uncertainty of our present knowledge as to why this organ is so peculiarly variable in its functional and anatomical responses to pathological conditions, for in just this feature lie the difficulties of the problems of experimental nephritis and Bright's disease in man.

With the perfused kidney of the frog the irritant may be introduced into one of two circulations and its action directed towards either glomerulus or tubule. But even under these controlled conditions the possibilities as to what may happen are enormous. There are anastomoses between the two systems the importance of which we have previously emphasized (5). The toxic substance may diffuse directly through the tissues or it may be excreted through the glomerulus and so reach the tubule. In this way its action may or may not be confined to the locality towards which it was directed. As a result any one or all of the following reactions may occur.

1. Arterial spasm.
2. Arterial dilatation.
3. Venous spasm.
4. Venous dilatation.
5. Increase in glomerular permeability.
6. Decrease in glomerular permeability.
7. Depression of tubular secretion.
8. Depression of tubular absorption.
9. Increase in tubular absorption.

And, based on the experiments of Richards (9) which will be mentioned later: There are therefore 511 immediate possible combinations with which the functional response of the kidney may begin, and as the reaction proceeds so that permutations of the factors can occur a tremendous number of possibilities develop for the further course of

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The functional variations in the glomerulus after toxic damage that we have demonstrated illustrate how useless it is to attempt any correlation from simple histological appearance. Such a glomerulus may be histologically plainly disrupted and so offer no barrier at all to the passage of fluid into the urine. Yet this increased permeability of its membrane may produce no increase in the volume of excreted water or other constituents of the urine, for vascular spasm, a concomitant response of the artery to the toxic agent, may reduce the supply of material available for excretion.

The mechanism by which tubular damage is intimately associated with the glomerular lesion is also illustrated. Any diffusible toxic substance that passes through the glomerular membrane into the tubular lumen comes in contact with the tubular epithelium and damage may result. We have found it therefore difficult to damage only the glomerulus with any of the substances we have used. Such a lesion of the tubule is however in no sense dependent or "secondary" to the glomerular lesion, but is part of the response of the kidney to the direct action of the toxin which has reached it by this circuitous route. There comes to mind the *nephrotische Einschlag* so common in "glomerular nephritis" and which on no very definite evidence, is considered by some to be a secondary and unimportant aspect of the kidney lesion in this form of Bright's disease.

Further evidence of the perils of deduction of function from morphology in our present state of knowledge is seen in our demonstration that the damaged tubule may absorb and yet fail to secrete or that the converse condition may exist. Until this paradox is solved, either by the localization of the two processes in different parts of the tubule or by some other explanation, a complete correlation of the histological and functional aspects of the kidney's response remains impossible.

SUMMARY AND CONCLUSIONS

1. A method of testing the frog's kidney by means of perfusion is described.
2. This is made possible by dissociating, as far as possible, from the total function of the organ the functions of its constituent parts.
3. The characteristics by which tubular, glomerular, and combined tubular-glomerular lesions may be recognized are described.

nephritis, but a general method is afforded for the study of the morphological aspects of pathological processes under controlled and simplified conditions. The tissues of organs thus studied are isolated from the complications of circulatory and nervous mechanisms, their environment is artificially and rigorously controlled, and conditions are therefore analogous in a certain degree to those which obtain in the study of tissue cultures.

In this communication we shall only describe the lesions as they affect the problem of experimental nephritis. The anatomical changes whose functional disturbances we have previously studied (1) will be described and compared with the anatomical lesions which we have found in the kidneys of the living frog (2).

Technique

The method of perfusion of the kidneys with oxygenated Locke's solution has been described. The urine, which is normal in volume, concentration, and constituents, is collected for examination by catheters placed in the ureters. Toxic agents in proper concentration may be introduced into the perfusion fluid and led by either the arterial or renal-portal venous circulations to the glomeruli or to tubules of the kidney. The urine is then collected as it is formed and examined.

The changes in its characteristics that result from the damage by the toxic agents, and the significance of these changes have been given in the preceding paper. At the end of such experiments the kidneys were removed, their gross appearance noted, and small pieces fixed in different solutions. For routine methods of staining Orth's fluid, 10 per cent isotonic neutral formalin, and absolute alcohol were used, while Bensley's fixative and Kolster's fluid were found suitable for the fixation of granular structures. The use of several fixatives is essential to avoid the dangers of artefact that may result from improper fixation. Formalin was found to be especially liable to such failure of proper action even when freshly neutralized and in isotonic solution. As stains, Delafield's hematoxylin and eosin, Van Gieson's mixture with hematoxylin, and the Mallory connective tissue stain were used. For mitochondria and other granules the Bensley and Altmann methods proved most satisfactory.

The Effect of Perfusion on the Tissues of the Kidney

Before proceeding to the study of the effects of toxic agents on the tissues it is obvious that one must first determine whether the perfusion as such produces structural changes. As we have stated the kidney may appear functionally normal even after 6 hours of perfusion and though this is strong presumptive evidence that no an-

EXPERIMENTAL NEPHRITIS IN THE FROG

III. THE EXTRAVITAL PRODUCTION OF ANATOMICAL LESIONS IN THE KIDNEY AND THEIR CORRELATION WITH THE FUNCTIONAL ASPECTS OF DAMAGE*

BY JEAN OLIVER, M.D., AND PEARL SMITH, M.D.

(From the Departments of Pathology of the Long Island College of Medicine, Brooklyn, and of Stanford University Medical School, San Francisco)

PLATES 27 to 30

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In the accompanying article experiments have been described in which different elements of the isolated perfused frog's kidney were damaged by various toxic agents injected into one or both of its circulations (1). The resulting functional disturbances were described and methods outlined for an analysis of the response of the kidney by means of dissociation of its various functions. In these experiments the use of the toxic agents was a means towards a special end. All that was required for our immediate purpose was the production of damage to either tubule or glomerulus so that the dysfunction of the kidney under such conditions might be tested. But with this purpose achieved a further question presents itself, and one of much broader significance. Do the lesions which develop in these isolated kidneys resemble in their morphological features the lesions which occur in the living frog when the same agents are administered *in vivo*? And are the pathological processes under artificial and living conditions so similar as to produce evidences of morphological identity? If this is so the significance of our experiments is greatly increased. Not only is interest added to the problem of experimental

* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacology and Chemistry, American Medical Association.

of the anatomical lesions which develop when the kidney, to judge by its functional response, has been damaged by toxic agents.

The Development of Morphological Processes as a Response to Extravital Damage

The experiments to be described are some of those whose functional aspects were examined in our preceding communication supplemented by others of similar nature. In them, the perfused kidney, after establishment of normal urine formation, was damaged by the introduction of some renal toxic agent into one or the other of its circulations and a series of type reactions demonstrated in which there were evidences of tubular or of glomerular dysfunction. At the end of the perfusion the kidney tissues were removed and fixed as described above for histological examination.

The Epithelial Lesions

The lesions in the epithelial structures were as a rule best studied in the mitochondrial preparations. In some instances the appearance of the tubule cells by the ordinary methods of staining was normal when their functional response after the introduction of the toxic substance had been found to be definitely abnormal. A study of the mitochondria in such specimens, however, showed that anatomical lesions were actually present.

1. *Cloudy Swelling*.—All the morphological characteristics of the process of cloudy swelling were found reproduced in the epithelium of the tubules of perfused kidneys following the introduction of toxic agents into the renal-portal circulation.

The toxic substances used included potassium bichromate, corrosive sublimate, urethane, and uranium nitrate. The histological appearance varied from initial stages of slight disarrangement and increase in size of the mitochondrial granules to extreme degrees where the cell body was swollen and filled with large closely packed granules which stained irregularly. The most typical pictures were those following potassium bichromate. An example is given in Experiment 2 of such an experiment showing the functional changes which occurred and the anatomical lesions that were found in the kidneys are shown in Fig. 6.

2. *Mitochondrial Changes Other than Cloudy Swelling*.—Beside the changes of cloudy swelling, which in the frog's kidney at least seem

atomical lesions have developed it does not preclude the possibility. The following experiment was therefore performed.

A frog's kidneys were prepared for perfusion with Locke's solution in the usual manner except that fine clamps were placed on the arteries which lead to the upper third and across the left kidney itself in such a way that removal of its upper pole allowed no fluid to escape. This tissue was excised and fixed in Orth's, Bensley's, and Kolster's fluids. The perfusion was now begun. The results are shown in Experiment 1. Three samples of kidneys were now available for histological study, the first specimen, that had not been perfused, the second that had been perfused for 40 minutes, and the third, that had been perfused for 1 hour and 10 minutes. Function was normal throughout the entire experiment. Histological examination of these three specimens stained with hematoxylin and eosin showed no differences between perfused and unperfused kidney tissue except that in the former the red blood cells had been washed away. A much more striking demonstration of the absence of any damage by the perfusion was seen in the mitochondrial preparations stained by either the Bensley or Altmann method. In all parts of the tubule the mitochondria had maintained their normal size and arrangement (Fig. 1). In the broad Segment II they appeared in the usual granular form with only slight filamentous arrangement while in Segment III the rodlet structures were perfectly preserved. This experiment was performed repeatedly with similar histological findings and was also confirmed by the histological examination of kidney's which had been perfused for other purposes.

Another and perhaps even more convincing demonstration of the harmlessness of the perfusion was discovered by chance.

Frogs often suffer from parasitic infection of their viscera which is manifested by the occurrence of focal areas of necrosis in the infected organs. A frog which had been prepared for perfusion was found to show such lesions in the kidneys. Nevertheless the experiment was continued and for $6\frac{1}{2}$ hours urine was formed, though not normally for later examination showed histological evidence of tubule and glomerular damage which was plainly the result of the infection. There were also present reparative processes of a regenerative character and many mitotic figures were found in the tubular epithelium and in the lining of the glomerular capsule. The point which interests us is that these mitotic figures, ranging through all stages from early prophase to late metaphase, were entirely normal in appearance and showed no effect from the $6\frac{1}{2}$ hours of perfusion. Even the achromatic spindle, one of the most delicate of cytological structures, was perfectly preserved in cells which had been bathed not only in the artificial perfusion fluid but also by the artificial urine (Fig. 3).

With the fact established that the method of perfusion does not of itself produce any change in the tissues we can proceed to the study

3. *Necrosis and Desquamation of Cells.*—The classical picture of cell damage and death was found in perfused kidneys stained with the simpler methods, such as Delafield's hematoxylin and eosin.

After the introduction of any of the toxic substances we have mentioned and after the development of functional evidences of damage that we have described, frank evidences of cell death and disintegration were found in Segment II. These included caryolysis, caryorrhexis, and pyknosis (Fig. 4). Of these changes the swelling of the nuclei with solution of the chromatin and the converse shrinkage with condensation were more commonly found than actual disruption and fragmentation. The protoplasm also showed both the swelling and the deep staining with eosin which is typical of the dead cell. Furthermore desquamation of these cells into the tubule lumen and all stages of their disintegration to a granular debris were seen, exactly as it was found in the kidneys that were damaged in the living animal. This desquamation was particularly prominent after the administration of corrosive sublimate (Fig. 2).

Cast formation in the strict meaning of the term was rarely seen in the sections even when a considerable amount of detritus was present in the lumen of the tubule. The early stages of their formation were evident, however, since incompletely consolidated material was found, cemented together by coagulated fibrin-like material.

Experiment 4 illustrates an example of a kidney damaged by corrosive sublimate in which necrosis and desquamation occurred (Figs. 2, 4) with the functional disturbances that resulted.

The Glomerular Lesions

1. *Lesions Producing a Simple Increase in the Permeability of the Glomerular Membrane.*—The simplest anatomical lesion observed in the glomeruli of living frogs after the administration of toxic substances was an apparent increase in the permeability of the glomerular membrane and the accumulation of precipitated material in Bowman's space along with more or less desquamation of the epithelial cells which line the capsule. Functionally this increased permeability was definitely demonstrated with the perfused kidney damaged *extra vivo* by the passage from the perfusion fluid into the urine of certain substances, such as gum arabic or colloidal dyes, which are held back under normal conditions (1, 3). In the glomeruli of kidneys thus damaged *extra vivo* the same lesions were produced as were observed *in vivo* with the exception that the absence of blood in the vessels of the perfused kidney precluded the possibility

to be in part a form of mitochondrial modification, all the other morphological appearances that have been described in damaged mammalian tissues have been encountered.

These changes were most pronounced in the broad Segment II of the tubules, and in cases of moderate damage were limited to this area. As the damage becomes more severe the lesion spreads both towards the neck of the tubule and into the narrower Segment III. The most commonly seen appearance was that of a disarrangement of the normal pattern with the formation of irregular agglutinated masses of granules clumped in some part of the cell. Often it was at the upper pole that the fused material was seen, but in some specimens the masses seemed to have formed at random in any part of the cell (Fig. 7). The breaking up of the filaments which are delicate structures in Segment II into granules was also observed, and in those kidneys which had been so severely damaged that Segment III was affected, the breaking up of the rodlets into irregular granular-like structures could be clearly followed. The granules thus formed were much finer and more delicate in appearance than the coarse ones of Segment II and were best studied in Bensley's preparation, a method which normally stains the rodlet of this portion of the tubule better than the Altmann method.

Quantitative changes were also found in the mitochondrial material, or at least variation occurred in the amount of it seen when stained by the ordinary methods used for its detection.

Some cells, especially those which presented the agglutinated masses just described, were almost completely filled with deeply stained material, while others showed little if any stained substance in any part of their protoplasm. The latter change seemed to be connected with what may be described as a lysis of the granules, for intermediate pictures of granules which were definitely faded to those which could hardly be seen on account of their pallor were commonly found. With this fading there often went an increase in the size of the individual granule. On the other hand increases in the amount of mitochondrial material were found; for example the increase in size and number of granules that has previously been described under the heading of cloudy swelling.

This statement of the changes that may occur in the mitochondrial elements of the cells is of necessity only a cursory summing up of the subject as it relates to the problem of experimental nephritis and a further discussion of the general aspects of the problem will be given in our discussion. An example of the functional changes in an experiment in which these changes (Fig. 7) were found is given in Experiment 3.

TABLE I
Functional Results of Experiments

	Arterial flow	Venous flow	Urine volume	Dye	Salt	Sugar	Gum
Experiment 1							
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	mg. per hr.		
10:35	Frog prepared. $\frac{1}{3}$ of left kidney removed						
10:50	Perfusion begun						
11:10-11:30	490	240	9.0	(P.R.) 0.54	36.0	0	
11:30	Left kidney removed after 40 min. perfusion						
11:30-12:00	400	240	5.4	0.33	25.0	0	
12:00	Right kidney removed after 1 hr. 10 min. perfusion						
Experiment 2							
10:30-10:45	600	600	4.4	(N.R.) 0.36	—	0	0
10:45-10:48	15 cc. 1/4000 potassium bichromate to tubule						
10:50-11:05	600	500	6.0	0.20	—	+	0
Experiment 3							
10:30-10:45	360	240	3.6	(N.R.) 0.14	—	0	
10:47-10:50	10 cc. 1/3000 potassium bichromate to tubules						
11:45-12:00	400	300	2.8	0.03	—	+	
Experiment 4							
11:45-12:00	360	440	4.8	—	24.0	0	
12:00-12:03	10 cc. of 1/2500 corrosive sublimate to tubules. Neutral red added to perfusion fluid						
12:15-12:30	200	480	8.0	Ft. tr.	48.0	++	
1:30- 1:45	160	480	1.2	Ft. tr.	9.0	++	
Experiment 5							
11:30-11:45	550	800	4.0	Trypan blue- pink, tr.	12.8	0	
12:00-12:05	10 cc. 1/7500 corrosive sublimate to glomeruli						
12:30-12:45	600	700	21.0	Trypan blue, perfect match to perfusion fluid	147.0	++	

of the escape of plasma, a source of fibrin, and of cells, either leucocytes or erythrocytes. It was therefore remarkable to find, after the administration of sublimate, uranium nitrate, potassium bichromate, or urethane, deposits of coagulated fibrinoid material identical in appearance and staining reactions to the deposits which had been found in the living animals whose vessels contained blood plasma (Fig. 8).

This fibrinoid material, as we have previously stated, resembles true fibrin in its fine fibrillar structure but differs from the latter in its reaction to Mallory's staining method. Mixed with this substance were found desquamated epithelial cells from Bowman's capsule. When tubule damage was associated with the glomerular lesion it was also found in the tubule lumen, where it permeated the masses of necrotic desquamated cells.

In this type of lesion the tuft appeared essentially normal. After the introduction of urethane the capillaries may be greatly dilated and since they contain no blood, the entire tuft appears washed out, but is otherwise normal. Experiment 5 illustrates such damage as produced by sublimate with the functional evidences of increased glomerular permeability. The two components of trypan blue were separated by filtration through the normal membrane so that the urine was tinged with pink, but after damage the dye appeared in the urine in the same blue color as was seen in the perfusion fluid (3). It will be noticed that there were also evidences of tubular dysfunction. Histologically marked evidence of damage was found in the tubular epithelium. The cells of Segment II showed not only mitochondrial alterations but even necrosis and desquamation.

2. Lesions Involving the Tissues of the Glomerular Tuft.—All the essential characteristics of the tissue lesions we have previously described in the glomeruli of living animals as an effect of toxic agents were observed in the perfused kidneys of the extravital experiments. After the introduction of any of these toxic substances into the arteries edema of the glomerular tuft was found, pyknosis of the cells of the tuft tissue, and even focal areas of necrosis with accumulation of nuclear debris. Fig. 9 showing the latter lesion may be compared with Fig. 3 of our previous publication to illustrate the similarity of the morphological appearances of the two lesions. Again the absence of blood plasma and blood cells in the vessels and the consequent impossibility of thrombosis in the extravital experiments produces certain differences which affect the general histological picture, but the essentials of the reactions of the tissues to the irritants

the tubular epithelium. We have already called attention to this intimate association of glomerular and tubular damage in our previous study of the functional lesions and have suggested a possible reason for its occurrence.

Lesions in the Interstitial Tissue

Since time is an important factor in the reaction of fixed tissues to an irritant and also because hemorrhage and exudation of lymphocytes from the circulating blood are a change commonly seen in them, a limitation exists to the production of anatomical responses in our extravital experiments. As a matter of fact, however, these reactions do not play a prominent part in experimental nephritis in the living frog. Intertubular edema was the common interstitial lesion encountered there and this was frequently found in the perfused kidneys of the extravital experiments. It followed the introduction of any of the toxic substances including snake venom, whose peculiar action extravitally we have just described, into the renal-portal and in a lesser degree into the arterial system. The lesion consisted of a dilatation of the capillaries with pouring out of fluid into and consequent distension of the tissues. The intertubular spaces were thus increased and some compression of the tubules resulted (Fig. 5). This illustration is taken from the same kidney, damaged by corrosive sublimate, which showed marked tubular lesions (Figs. 2, 4). Along with such interstitial lesions were found varying degrees of the glomerular and the tubular damage.

DISCUSSION

Little discussion is needed in regard to the specific problem with which this communication deals. It has been shown that the functional abnormalities whose presence was demonstrated in the preceding article are accompanied by structural changes in the tissues of the kidney. Both glomeruli and tubules had been altered by the experimental procedure and as we have previously shown in the examples we have given above, the two aspects of damage may be correlated to a reasonable degree. For instance, we might cite those experiments in which functional evidences of combined glomerular and tubular damage were found after the administration of a toxic

TABLE I—*Concluded*

	Arterial flow	Venous flow	Urine volume	Dye	Salt	Sugar	Gum
Experiment 6 (Continuation of Experiment 2)							
	cc. per hr.	cc. per hr.	cc. per hr.	mg. per hr.	mg. per hr.		
11:25-11:28	15 cc. 1/4000 potassium bichromate to glomeruli			(N.R.) 0.11	—	+	+
11:30-11:45	440	500	4.0	0.05	—	+	++
11:45-12:00	500	400	2.8		—	+	++
Experiment 7							
11:00-11:15	500	400	6.0	0.12	30.0	0	0
11:16-11:19	15 cc. 1/20,000 venom to tubules						
11:20-11:30	480	300	4.8	0.10	24.0	0	0
11:30-11:35	15 cc. 1/20,000 venom to glomeruli						
11:45-12:00	400	400	3.2	0.04	19.0	0	+
12:15-12:30	400	400	2.4	Tr.	—	+	++

are closely reproduced. Such necrosis, as has been stated, may follow any of the substances we have used, but the lesion was most striking after potassium bichromate. The details of the functional damage in a typical experiment are given in Experiment 6 from which Fig. 9 was taken.

An interesting result was obtained when snake venom was introduced into the arterial circulation of the perfused kidney.

In the living animal we were unable to produce any definite anatomical lesion with this substance but definite functional damage was found in the extravital experiments. When examined histologically all of the glomerular lesions we have just described were found including even the severe reaction of necrosis. Experiment 7 shows the details of the functional damage of a typical example. Beside the anatomical lesions in the glomeruli there were also morphological changes in the tubular epithelium. In the broad Segment II cloudy swelling and mitochondrial disarrangement were found.

A solution of cantharidin in acetic ether also damaged the tissues, but the complication of the irritating solvent prevented definite conclusions as to the significance of the damage.

As several of our experiments have shown, along with the lesions in the glomeruli there were frequently found anatomical lesions in

The anatomical changes that were noticed after the action of the toxic agent were similar to those in the nephritic animal. The following features may be emphasized.

1. There was an identity in the histological picture observed after the action of the drug in both the isolated kidneys and those taken from the poisoned living animals. The mitochondrial alterations, cloudy swelling, and agglutination, the nuclear changes of pyknosis, caryorrhexis and caryolysis, necrosis and desquamation of the cells into the tubular lumen, the formation of casts, the occurrence of necrosis in the glomeruli and of edema in the interstitial tissue, all were reproduced in the isolated kidney as they occurred in the kidney of the living frog. A striking example of this similarity may be seen by comparing Fig. 2 of the present study with Fig. 1 of our previous description of lesions that develop in the nephritic animal (2). In both cases the toxic agent was corrosive sublimate. The two illustrations are so similar that they might be easily interchanged without incongruity.

2. A certain "specificity" of action which had been noted in the type of lesion produced by certain toxic agents in the living frogs was also noticed in the extravital experiments. Cloudy swelling was produced in its best example by the action of potassium bichromate. This was true in the nephritic frogs and as Ophüls has shown, the same is true in the production of this change in the mammalian kidney (4). Necrosis with desquamation of coagulated dead cells into the lumen of the tubule which is thus filled with debris was found best exemplified in the extravital experiments after corrosive sublimate. The same features characterize the kidneys of frogs which had been poisoned by this substance, and is true even of the human "sublimate kidney." The glomerular lesions of edema and necrosis which were found in the living animals after bichromate were also best developed after the administration of this substance extravitally.

3. The localization of tubular lesions of lesser degree to the epithelium of the broad Segment II of the tubule which was such a pronounced feature of the action of the toxic agents in the living animal, was also plainly evident in the extravital experiments. Examples were seen where the damage was only in this part of the tubule, the neck and Segment III remaining essentially normal. In cases of

substance to the glomeruli. In the histological preparations of these kidneys there were also found structural changes in both glomeruli and tubules. We shall not insist on this phase of the problem, however, as it is obvious that the chief value of the method in the matter of correlation as well as its rigorous test will come when it is applied to the kidneys of frogs which have developed a nephritis under living conditions. This work is now in progress.

Of greater significance than this limited phase of our experiments is the demonstration that it is possible to produce in isolated organs and under conditions of artificial control structural changes that are morphologically identical to those which characterize the pathological processes as they occur in the living animal. It may be emphasized that these alterations cannot be considered mere artefacts due to abnormalities of osmotic pressure or reaction in the fluid which forms the nutrient supply of the tissues. The organs were living under "normal" though artificial conditions, for they were performing their highly specific and complex functions in a normal manner when the toxic agent was introduced in a concentration that approximated what might occur in the living animal. There then occurred an alteration of function that corresponded, as we shall show in detail later, with those observed in the kidneys of living animals when they were subjected to the same treatment. And finally when the structural changes in the perfused kidneys were examined histologically and compared to those which we had studied in living frogs (2) the same similarity was found. Since this last point is the one with which the study is concerned, we shall summarize the findings which bear upon it.

It was demonstrated that the method of perfusion, when successfully performed as determined by functional criteria, produced no structural changes of an abnormal character in even the finest and most delicate of cell structures. The mitochondrial elements were perfectly preserved and even the fibrils of the achromatic spindle of mitotic figures were not damaged. Epithelial cells were not swollen, the brush border, a difficult histological structure to preserve, was intact, the cilia in the neck of the tubule were clear and distinct, and so we might continue to enumerate all of the elements of the kidney tissue.

problem, for these last mentioned phenomena are not important in the lesions that develop in the nephritic frog. If necessary the method could obviously be modified by the use of properly prepared blood for the perfusion.

In spite of these drawbacks the use of the extravital method in some phases of our problem has allowed us to study more clearly the reaction of the tissues to the toxic agent than was possible under living conditions. It will be remembered that we were unable to produce satisfactory lesions in the living frog by the use of snake venom. In the perfused kidney abnormalities developed regularly after its introduction into either of the circulations. The anatomical lesions though they cannot be compared to any in the living frog, are strikingly similar to those which have been described in mammals which are susceptible to snake venom intoxication, namely, slight epithelial degenerations and more striking damage to the glomeruli. The earlier stages of cast formation are also better shown than in the kidney of the nephritic animal for the consolidation of desquamating disintegrating cells into definite precursors of well formed casts is easily seen. The fibrinoid material seems to be an important factor in the binding together of such heterogenous material.

It seems reasonable to suppose therefore that the extravital method used under the conditions of our experiments will prove valid and useful for the investigation of many problems. For example, it is easily possible to stain the functioning kidney extravitally* and to modify at will the conditions under which the reactions are occurring. This procedure has been used by us in the problem of experimental nephritis and will be reported at a later time.

Another use of the method that our specific problem has indicated is in the study of the mitochondrial elements of the cell. Our observations on these structures were of necessity subordinated to those aspects which concern experimental nephritis, but are being continued in a more detailed study. The vexed question of correlation of hyperactivity in the kidney with mitochondrial change and

* The need of the term extravital is evident when used in reference to staining, as neither the term vital staining or supravital staining can be applied to the process by which the living kidney stains itself under the conditions we have described.

severe damage the extension of the lesion into these portions of the tubule was, as in the nephritic animal, evident

4. The peculiar substance resembling fibrin which we have described in the glomerular spaces of animals poisoned during life was also found in the extravital experiments. Its physical density was less than that observed in the vital experiments when 48 to 72 hours elapsed before the death of the animal, but its reactions to the Gram and Mallory stain were identical. But in the extravital experiments the source which had been considered so obvious in the living experiments was absent, since the vessels of the perfused kidney contained no blood or plasma. This material must therefore be derived from the tissues. Not only the glomerulus is a source of this material, but since the tubules were found to contain it as well, some may have passed back from the lower lumen and coagulated in Bowman's space or on the glomerular tuft.

Contrasting with these similarities, on the other hand, are certain factors which limit the formation and development of the pathological processes in the extravital experiments. We have already mentioned one; the time factor. The poisoned frog lives from 24 to 72 hours before death brings to a close the development of the anatomical lesion. The extravital experiment can be prolonged at best 8 to 10 hours and only the earlier stages of many processes can therefore be observed. Autolytic phenomena which play such an important part in the changes which are observed in necrotic cells are thus limited in their degree, though as we have shown, they are sufficiently advanced to be clearly recognizable. The reaction of fixed tissue cells to an irritant is also largely excluded by the time element, for these are typically of a proliferative character. The observation of numerous and active mitotic figures, however, leads one to suppose that such changes are entirely possible under the conditions of the experiment. Another factor which limits the response of the tissues is the absence of circulating blood, both cells and plasma, in the vessels. There can be no hemorrhage, a prominent feature in the glomerular lesions of the living frog, nor any leakage of protein-containing fluid as a transudate from vessels. No exudation of leucocytes can occur. These handicaps to the production of analogous lesions under vital and extravital conditions are not serious in our

EXPLANATION OF PLATES

PLATE 27

FIG. 1. Tubules of the kidney of Experiment 1 after 1 hour and 10 minutes normal perfusion stained by the Altmann method after Kolster's fixation. The mitochondria of Segment II are shown best in the long central tubule; those in the section to the left are insufficiently differentiated. Note that the granules are small, clustered about the nuclei, even in size, and entirely normal in every way. 600 \times .

FIG. 2. General view of the damage produced extravitally by corrosive sublimate in Experiment 4. Bowman's space contains granular material and desquamated cells. There is extensive necrosis and desquamation of the cells of the tubules, especially of the broad Segments II in the lower half of the figure. Segments III, though also damaged, are better preserved. Pyknosis of the nuclei is evident. The intertubular capillaries since they contain clear Locke's solution instead of blood appear empty, yet the general picture is identical to that of the lesion produced by sublimate in the living animal. Cf. Fig. 1, Oliver and Smith (2). Hematoxylin and eosin after Orth's fixation. 140 \times

PLATE 28

FIG. 3. Perfectly preserved mitotic figures from a kidney which was perfused for $6\frac{1}{2}$ hours. The delicate fibrils of the achromatic spindles are difficult to reproduce photographically but are well preserved. Delafield's hematoxylin and eosin. 1200 \times .

FIG. 4. Nuclear changes in the epithelium of Segment II of the kidney of Experiment 4 after the administration of corrosive sublimate. The desquamated epithelial cells fill the tubule lumen. To the left dense pyknotic nuclei are seen; to the right and above the swollen appearance of caryolysis is evident. Occasionally there may be found a nucleus in caryorrhexis. Orth's fixation, hematoxylin and eosin. 670 \times .

FIG. 5. Interstitial edema in the kidney of Experiment 4 following damage by corrosive sublimate. The intertubular capillaries are dilated and since they contain only Locke's solution, appear empty. The connective tissue fibrils and cells are separated by the infiltrating fluid which has escaped from the vessels. The tubular epithelium is damaged, as evidenced by the pyknosis of the nuclei. Orth's fixation, hematoxylin and eosin. 170 \times .

PLATE 29

FIG. 6. Cross-sections of tubules, chiefly Segment II, from Experiment 2 after the action of potassium bichromate the kidney being fixed in Kolster's fluid and stained by the Altmann method. Extreme cloudy swelling of epithelial cells of Segment II is seen. The swollen cells are filled with swollen irregularly staining granules which in some places are fused into what appears as a solid mass of material. 440 \times .

its relation to the pathological change of cloudy swelling would also appear to be an appropriate subject for investigation by the extravital method. The method in this way supplements and extends the method of Lewis and Lewis (5) whose examination of the mitochondria under experimental conditions in tissue culture cells first brought a sense of reality to the problem. In their work the cells were of a simple undifferentiated type and the experimental modifications were those of simple changes of osmotic pressure and reaction of the medium. In the extravital preparations of the kidney we are dealing with highly specialized cells that are performing in a normal manner their specific function under conditions that are amenable to control. It may be that its use may afford an answer to the cogent criticism of Cowdry (6) when he states in speaking of mitochondrial changes that "we have a plethora of observations but no new experimental method has brought us noticeably nearer to a solution of the puzzle."

SUMMARY AND CONCLUSIONS

1. It is possible to produce in the perfused frog's kidney an experimental nephritis which is anatomically similar to that which develops in the living animal.
2. The functional effects of these anatomical alterations may be examined by a method previously described.
3. The correlation of the two aspects of damage, anatomical and functional, is more certain under such conditions than in the living animal.
4. The value of the extravital method in general problems is indicated by our brief consideration of mitochondrial changes.

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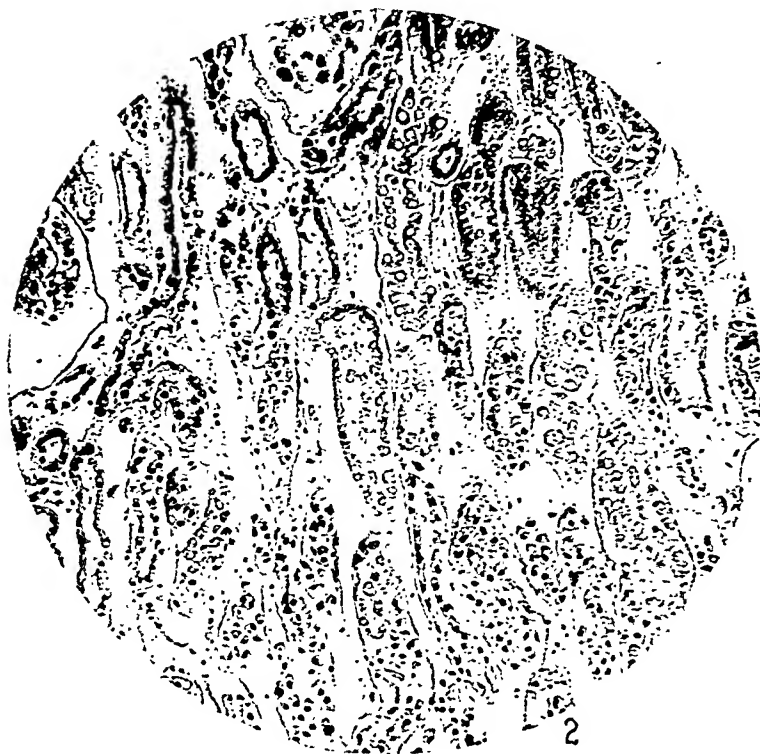
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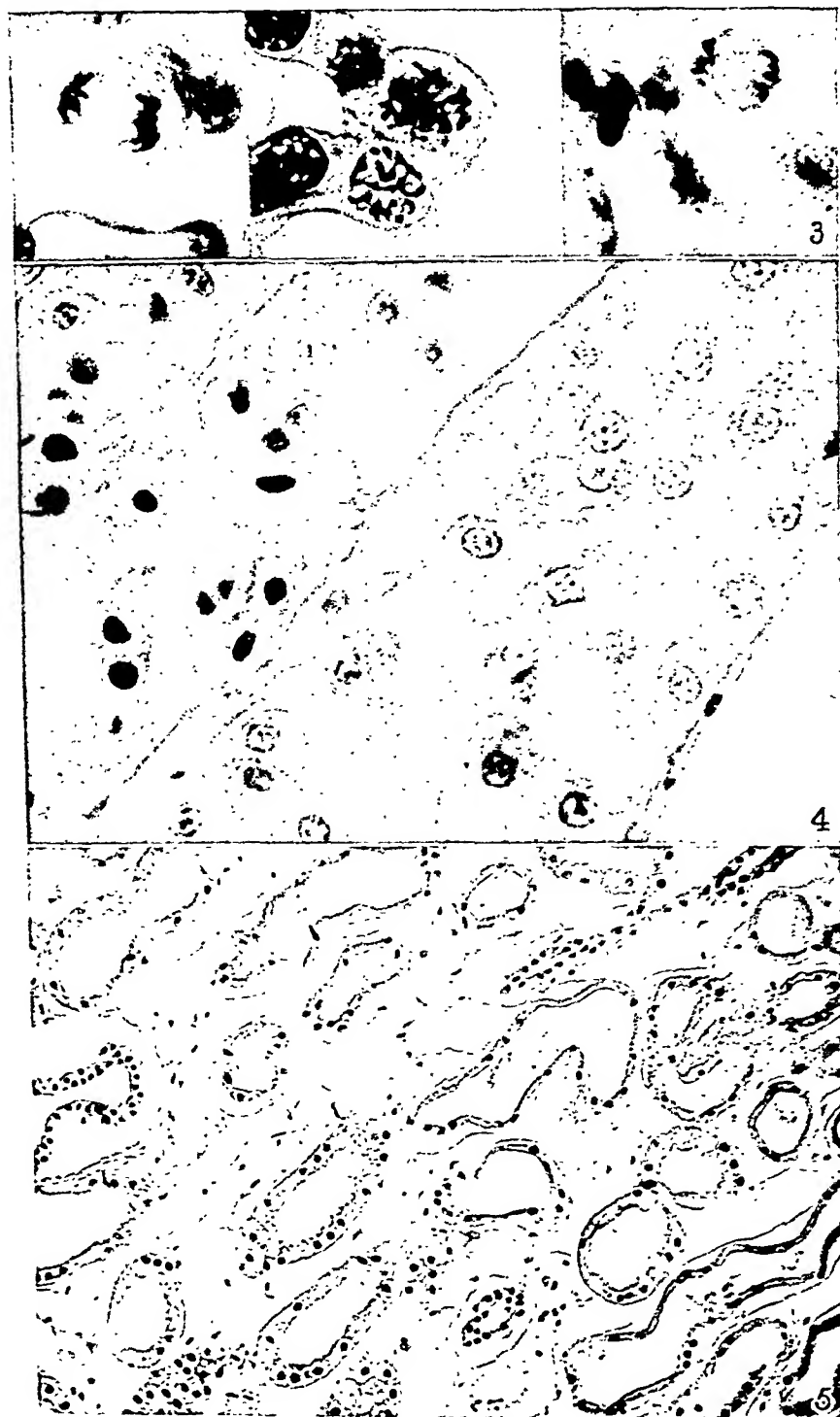
FIG. 7. Mitochondrial changes following potassium bichromate in the kidneys of Experiment 3. The lesions are more severe. In the lower insert individual granules may be seen in the process of agglutination. In the larger figure the mitochondria have fused into large masses. Note the irregularity of their distribution, some cells being empty, others completely filled with irregular clumps of dense material. Altmann stain, Kolster's fixation. 440 \times .

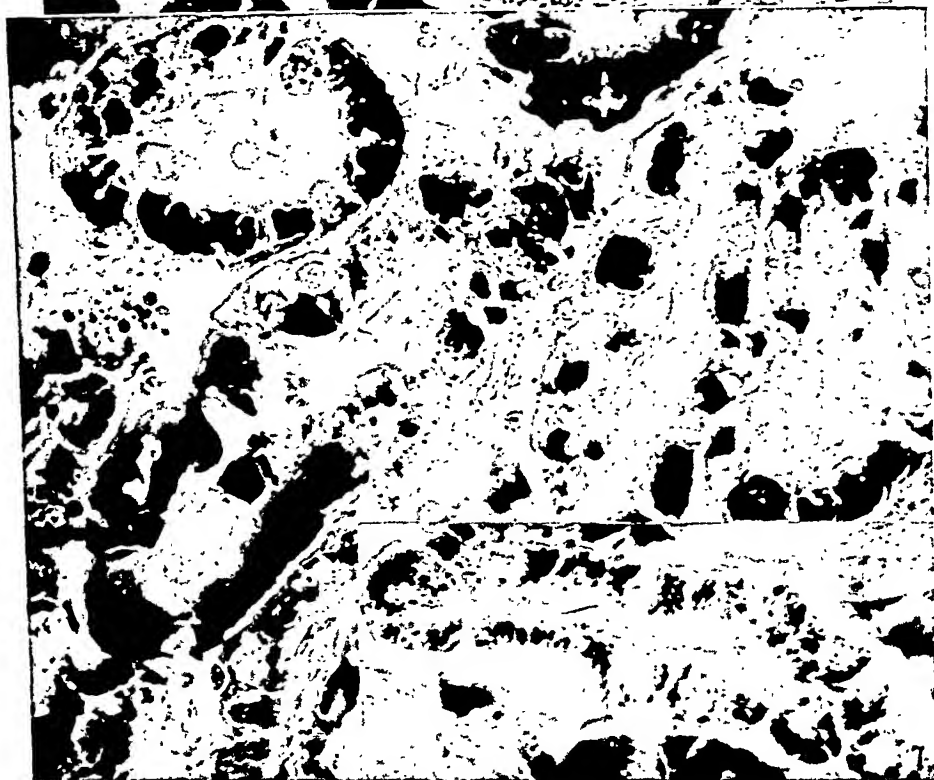
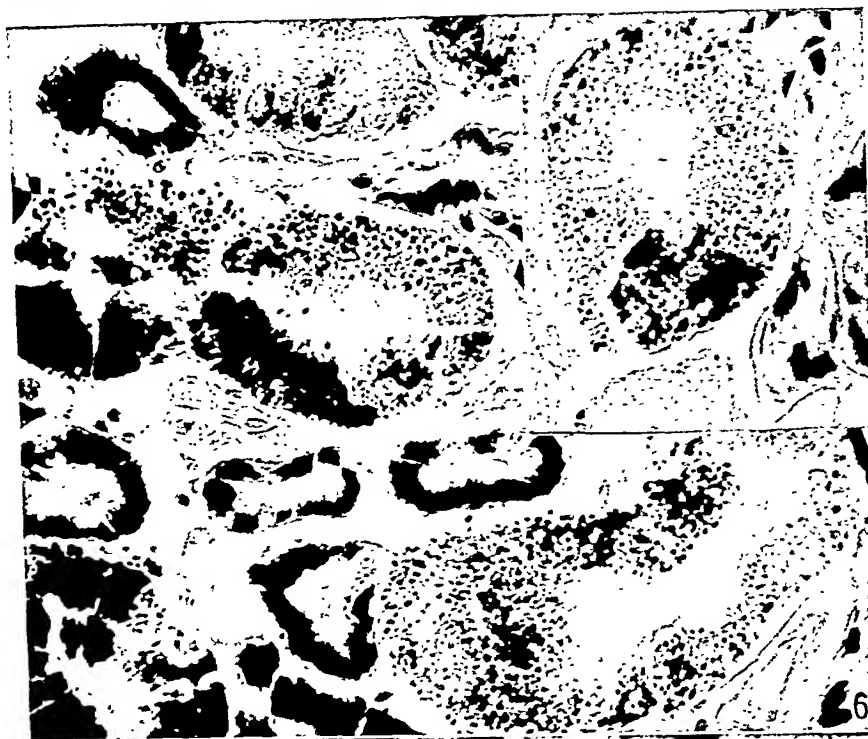
PLATE 30

FIG. 8. Fibrinoid material which resembles fibrin in its structure but differs in its reaction to staining methods is seen in the capsular space following administration of corrosive sublimate to the glomeruli of the perfused kidney of Experiment 5. Except for the density of the material its appearance is essentially similar to that found in the glomeruli which were damaged in living animals. Cf. Fig. 4, Oliver and Smith (2). Orth's fixation, Mallory's stain. 320 \times .

FIG. 9. The glomerular tuft in the perfused kidney of Experiment 6, the result of the introduction of potassium bichromate into the arterial system. There is not only edema, but frank necrosis and pyknosis of tuft nuclei. The lesion is therefore essentially similar to that found in kidneys of nephritic animals, the difference in the histological picture being largely the result of the washing out of the blood from the glomerular capillaries by the perfusion fluid. Cf. Fig. 3, Oliver and Smith (2). 340 \times .









cocci. Inasmuch as all of these fractions contained small amounts of nitrogen it could not be stated definitely that anaphylactic shock had been produced by carbohydrate hapten alone. Similar results, however, were obtained by Avery and Tillett (5) who injected into passively sensitized guinea pigs protein-free specific carbohydrates of *Pneumococcus* I, II, and III. (The fractions from Types II and III were nitrogen-free as well.) The anaphylactic reaction was type-specific. Attempts to induce active sensitization by injection of the polysaccharides were uniformly negative. Using a specific carbohydrate obtained from the tubercle bacillus and containing 0.3 per cent nitrogen, Enders (6) obtained lethal anaphylactic shock in passively sensitized guinea pigs and also in guinea pigs actively sensitized with dead tubercle bacilli. The minimum lethal dose of carbohydrate was 0.5 mg. for the passively sensitized animals and 2 mg. for the actively sensitized. While the present work was in progress Kurotchkin and Lim (7) reported the production of anaphylactic shock in guinea pigs actively sensitized to *Monilia pinoyi* and *M. psilosis*. Fatal shock followed the injection of 1 mg. of *M. pinoyi* soluble specific substance into guinea pigs actively sensitized with the homologous organism. Sublethal shock was obtained in the case of *M. psilosis* with a dose of 2 mg. of the corresponding substance.

EXPERIMENTAL

Material Employed.—The organisms are those used previously (1), namely: (1) A *Monilia albicans* isolated from an interdigital erosion and identical culturally and morphologically with *Monilia psilosis*. (2) *Monilia psilosis*, Ashford. (3) A monilia similar to *M. parapsilosis*. (4) A strain of *Willia anomala*. (5) A stock strain of *Saccharomyces cerevisiae*.¹

Antisera were prepared in rabbits by intravenous injections of heat-killed organisms grown in Sabouraud's honey broth. The sera in dilutions of 1:500 agglutinated the homologous organisms, and in addition a number of cross-agglutinations were evident (1).

A water-soluble fraction was prepared from each of the organisms. Protein tests on these fractions are doubtful or negative (1), although small amounts of nitrogen are present² (Table I), but they react strongly to the Molisch test, yield reducing sugar on hydrolysis, and are precipitated in high dilution by the homologous antisera. Quantitative solutions of the fractions were made up in sterile salt solution.

¹ We are indebted to Miss R. W. Benham of the Medical Mycology Laboratory of the Department of Dermatology, College of Physicians and Surgeons, Columbia University, for the original cultures and for numerous helpful suggestions.

² Dumas micro nitrogen determinations were kindly done by the Department of Biochemistry, College of Physicians and Surgeons, Columbia University.

HYPERSENSITIVENESS TO SOLUBLE SPECIFIC SUBSTANCES FROM YEAST-LIKE FUNGI

I. ANAPHYLAXIS

By H. D. KESTEN, M.D., AND E. MOTT, M.D.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia
University, New York)

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A previous report (1) from this laboratory was concerned with the preparation and properties of a water-soluble fraction, essentially a polysaccharide, from each of five yeast-like fungi. Tested by direct precipitation against the corresponding antisera these polysaccharides exhibited only partial specificity, cross-precipitin reactions being frequent. By absorption of precipitin on the intact mycotic bodies, however, a relatively high degree of specific precipitability was demonstrated for the soluble substances.

The present paper deals with (1) an attempt to induce active sensitization to one of these soluble substances by injection (a) of the substance itself, (b) of the killed homologous organism; (2) the production with the several fractions of anaphylactic shock in guinea pigs passively sensitized with homologous fungus antiserum; and (3) a comparison of the specificity of the anaphylactic phenomenon with that of the precipitin reaction.

Tomcsik (2), using a soluble specific carbohydrate substance obtained from *B. lactis aerogenes*, induced fatal anaphylactic shock with intravenous doses as small as 0.033 mg. in guinea pigs passively sensitized with homologous rabbit antiserum. Tomcsik and Kurotchkin (3) subsequently reported similar results with carbohydrate haptens from *B. lactis aerogenes*, the pneumobacillus, and a yeast. Attempts to produce active sensitization against the *B. lactis aerogenes* specific substance by repeated inoculation of living and dead bacteria were unsuccessful. Lancefield (4) produced fatal shock in guinea pigs passively sensitized with antistreptococcus serum using a carbohydrate fraction isolated from strepto-

antigenic when used in this way. It is possible that a single sensitizing dose would have been more effective, but Avery and Tillett (5) were unable to induce active sensitization in this fashion with pneumococcus polysaccharides.

B. A similar attempt to induce active sensitization to the soluble substance was carried out, using killed monilia bodies as the sensitizer.

250 gm. guinea pigs were given 10 consecutive daily intraperitoneal injections of 0.2 cc. of a 5 per cent suspension of heat-killed washed *Monilia psilosis* organisms grown 72 hours in Sabouraud's broth. (The suspension consisted almost entirely of budding forms with relatively few mycelia.) 21 days after the last injection the animals were given an intravenous or intracardiac shocking dose of from 0.5 to 2 mg. of the homologous soluble substance. Three animals of the series were given a suspension of ground or whole monilia bodies in a dose representing approximately 2 mg. of polysaccharide.

The results are presented in Table III. Of the eight animals given polysaccharide in the series, all but one showed evidences of anaphylactic shock immediately following the shocking injection. Two of them died an anaphylactic death and at autopsy showed the characteristic marked pulmonary emphysema associated with bronchial spasm. The three animals given a shocking dose of monilia bodies also showed symptoms of anaphylactic shock, and one died.

These findings confirm and extend those of Kurotchkin and Lim (7), who recently produced sublethal shock by intravenous administration of a *Monilia psilosis* soluble substance in guinea pigs previously sensitized with the dead fungi. Their inability to produce fatal shock may have been based on a lower degree of sensitization due to less intensive preliminary dosage, or on a less potent soluble fraction.

The possibility that the shock is caused by protein contamination of the polysaccharide fraction is remote. Unless all the nitrogen present, 0.84 per cent, represents protein nitrogen (an improbable assumption in the light of the negative protein tests), the amount of protein in a 2 mg. dose of soluble substance would be rather less than the minimum lethal anaphylactic dose of purified protein, 0.05 or 0.1 mg., as given by Wells (9). The results in passively sensitized animals provide further evidence in favor of the conclusion that the shock is induced by polysaccharide independent of possible protein contaminant.

Active Sensitization.—A. An attempt was made to actively sensitize animals by injection of one of the soluble substances.

Using Holobut's method (8), guinea pigs of about 250 gm. weight were given on 10 consecutive days intraperitoneal injections of 10 mg. of the soluble substance from *Monilia psilosis*. 21 days after the last injection the animals were tested by intravenous, intracardiac, or intraperitoneal injection of from 0.25 to 4 mg. of the same soluble substance.

TABLE I
Properties of Polysaccharide Fractions

Soluble substance from organism	N	Highest dilution giving Molisch reaction	Highest dilution giving precipitate with serum of rabbit immunized against homologous organism
	per cent*		
1. <i>Monilia albicans</i>	0.85	1:1,000,000	1:1,000,000
2. <i>Monilia psilosis</i>	0.84	1:1,000,000	1:1,000,000
3. <i>Monilia parapsilosis</i>	0.65	1:1,000,000	1:1,000,000
4. <i>Willia anomala</i>	1.48	1:1,000,000	1:1,000,000
5. <i>Saccharomyces cerevisiae</i>	1.78	1:1,000,000	1:1,000,000

* Substances dried over sulfuric acid, analyzed by Dumas micro method.

TABLE II
Absence of Active Anaphylaxis to Monilia psilosis Soluble Substance in Guinea Pigs Sensitized with the Soluble Substance

Guinea pig No.	Sensitizing dose <i>M. psilosis</i> polysaccharide, l.p.	Interval	Shocking dose <i>M. psilosis</i> polysaccharide	Result
		days	mg.	
83	10 daily 10 mg. each	21	2.0 i.c.	No effect
84	10 " 10 " "	21	2.0 i.v.	" "
85	10 " 10 " "	21	4.0 i.p.	" "
86	10 " 10 " "	21	0.25 i.v.	" "

The data are presented in Table II. Although the number of animals was limited because of the difficulty in obtaining the polysaccharide in quantity, the failure of any animal to react following the injection of the soluble substance indicates a lack of sensitivity, and one may tentatively conclude that the soluble substance is non-

Guinea pigs of approximately 250 gm. weight were sensitized by intraperitoneal injections of 0.5 or 1.0 cc. of serum from a rabbit immunized against *Monilia psilosis*. 24 hours later each pig received 0.01 to 1.0 mg. of the homologous soluble substance intravenously or intracardially. Control animals were injected with normal rabbit serum and tested with the same soluble substance.

The results appear in Table IV. Of the eight animals sensitized with *Monilia psilosis* antiserum, six died in anaphylactic shock in $3\frac{1}{2}$

TABLE IV

Passive Anaphylaxis to Monilia psilosis Soluble Substance in Guinea Pigs Sensitized with Monilia psilosis Rabbit Antiserum

Guinea pig No.	Sensitizing dose monilia antiserum i.p.	Interval	Shocking dose <i>M. psilosis</i> polysaccharide i.v. or i.c.	Result	Autopsy
	cc.	hrs.	mg.		
146	1.0	24	1.0 i.v.	† 5 min.	Typical
149	1.0	24	0.75 "	† 4 "	"
148	1.0	24	0.25 "	Cough, snuffles, dyspnea, fur ruffled, legs weak	—
60	0.5	24	0.2 "	† $3\frac{1}{2}$ min.	Typical
17	0.5	24	0.1 i.c.	† 4 "	"
204	0.5	24	0.05 i.v.	† $3\frac{1}{2}$ "	"
63	0.5	24	0.01 "	† $3\frac{1}{2}$ "	"
62	0.5	24	0.01 "	Cough, fur ruffled, bucking, dyspnea, urination, defecation	—
	<i>Normal serum</i>				
73	0.75	24	2.5 i.c.	No effect	—
50	0.5	24	3.0 "	" "	—
37	0.5	24	2.0 i.v.	" "	—
36	0.5	24	0.5 i.c.	" "	—
93	0.5	24	0.1 i.v.	" "	—
213	0.5	24	0.1 "	" "	—

to 5 minutes. At autopsy they showed the characteristic marked pulmonary emphysema. The other two animals exhibited definite symptoms of shock, including coughing, bucking, scratching of nose, ruffling of fur, dyspnea with deep abdominal breathing, urination, and defecation. When retested the following day, they were antianaphylactic. Control animals were symptom-free. The minimum lethal

Passive Sensitization.—As already reported (1), the rabbit antisera prepared against each of the five yeast-like organisms contain precipitins for the homologous soluble substance, and also for one or more of the heterologous substances. Separation of the precipitins can be

TABLE III

Active Anaphylaxis to Monilia psilosis Soluble Substance in Guinea Pigs Sensitized with Monilia Organisms

Guinea pig No.	Sensitizing dose killed <i>M. psilosis</i> i.p.	Interval	Shocking dose <i>M. psilosis</i> polysaccharide i.v. or i.c.	Result	Autopsy
		days			
54	10 daily 0.2 cc. 5% suspension	21	0.5 mg. i.v.	Cough, dyspnea, convulsions	—
55	" "	21	1.0 " "	Fur ruffled, cough, scratches nose	—
57	" "	21	2.0 " "	Fur ruffled, cough, dyspnea	—
75	" "	21	1.0 " "	Cough, dyspnea, restlessness, urination, defecation, ataxia	—
76	" "	21	1.0 " i.c.	No effect	—
77	" "	21	2.0 " i.v.	† 4 min.	Typical
78	" "	21	0.5 " "	Cough, marked dyspnea, ataxia, inability to stand	—
79	" "	21	2.0 " "	† 3½ min.	Typical
80	" "	21	0.2 cc. 20% suspension ground <i>M. psilosis</i> i.v.	Cough, dyspnea, defecation, pulmonary edema	—
81	" "	21	" "	† 4 min.	Typical
82	" "	21	0.4 cc. 10% suspension whole <i>M. psilosis</i> , i.v.	Dyspnea, urination, defecation	—

effected by absorption with the organisms. It was deemed of interest to sensitize animals passively with each of the five antisera and to investigate the ability of the several polysaccharide fractions to induce anaphylactic shock. To test each soluble fraction against each antiserum involved, accordingly, the use of twenty-five series of guinea pigs. A complete protocol of one series follows.

SOLUBLE SUBSTANCES FROM FUNGI

Anaphylactic Reactions to Fungus Soluble Substances

24 hrs. later. Shocking dose of soluble substance prepared from	Guinea pigs sensitized	
	1. <i>Monilia albicans</i>	2. <i>Monilia psilosis</i>
1. <i>Monilia albicans</i>	0.05 mg. ++ 0.05 mg. ++ 0.03 mg. ++ 0.02 mg. ++	1.0 mg. ++ 0.1 mg. ++ 0.05 mg. ++ 0.05 mg. + 0.05 mg. +
2. <i>Monilia psilosis</i>	1.0 mg. ++ 0.1 mg. ++ 0.05 mg. ++ 0.05 mg. + 0.02 mg. +	1.0 mg. ++ 0.75 mg. ++ 0.25 mg. + 0.2 mg. ++ 0.1 mg. ++ 0.05 mg. ++ 0.01 mg. ++ 0.01 mg. +
3. <i>Monilia parapsilosis</i>	0.05 mg. ++ 0.05 mg. ++ 0.02 mg. +	3.0 mg. ? / 0.075 mg. H ++ 2.5 mg. -0 / 0.075 mg. H ++ 1.0 mg. ? / 0.1 mg. H ++ 1.0 mg. -0 / 0.75 mg. H ++
4. <i>Willia anomala</i>	2.5 mg. -0 / 0.05 mg. H ++ 0.5 mg. -0 / 0.05 mg. H +	2.0 mg. -0 / 0.1 mg. H ++ 1.0 mg. -0 / 0.03 mg. H ++ 0.5 mg. -0 / 0.05 mg. H ++
5. <i>Saccharomyces cerevisiae</i>	2.0 mg. -0 / 0.05 mg. H + 1.0 mg. -0 / 0.1 mg. H ++ 0.5 mg. -0 / 0.05 mg. H ++	2.0 mg. -0 / 0.05 mg. H ++ 1.0 mg. -0 / 0.05 mg. H ++ 0.5 mg. -0 / 0.075 mg. H ++

++ = Anaphylactic death in 3 to 5 minutes.

+ = Sublethal anaphylactic reaction.

/ = Retested but with homologous (H) soluble substance.

* = Retested 3rd day after sensitization.

dose of soluble substance was 0.01 mg. Contaminating protein as a possible factor in the production of shock can be definitely excluded, as this dose of soluble substance is less than the usual minimum lethal anaphylactic dose of pure protein (0.1 or 0.05 mg.).

A similar series of guinea pigs was sensitized to each of the five antisera and tested 24 hours later with the homologous soluble substance. Further, animals sensitized with each antiserum were tested with each of the four heterologous soluble substances.

The dose of sensitizing serum was 0.5 cc. in all but an occasional instance when 1.0 cc. was given. The shocking dose of soluble substance varied from 0.01 to 2.5 mg. Any animal which did not show evidence of shock following heterologous soluble substance was retested, usually on the same day, with the homologous, in order to be certain that sensitization had been effective. An occasional such animal failed to react to the homologous substance and was rejected from the series. Occasionally, also, an animal given the shocking dose into the heart died with a hemopericardium. Such an animal was also rejected. Most of the injections of soluble substance were given into a superficial vein of a hind leg.

Table V contains in abridged form the data of these experiments. Anaphylactic death was regularly obtained in animals sensitized with each of the five antisera and shocked with the respective homologous soluble substance. The minimum lethal dose of soluble substance was 0.1 mg. or less. The table includes the results of retesting with homologous substance on the same day those animals negative to heterologous substance. Most of the animals that recovered from shock were also retested a day or two later and found to be refractory.

As Table V indicates, in many instances a heterologous soluble substance also induced anaphylactic shock in a sensitized animal. This is more readily appreciated from Table VI, columns A, which are a summary of Table V, each of the twenty-five sets of animals being recorded as a single result. Because the minimum lethal dose of homologous substance was 0.1 mg. or less, the results are indicated as ++, representing series in which 0.1 mg. or less induced lethal shock, or + for series in which more than 0.1 mg. of soluble substance was required to kill.

In view of the more or less generally accepted identity of precipitin and sensitizing antibody (10) it is of interest to compare the above passive anaphylactic reactions with the precipitin potency of the anti-

sera used to sensitize the animals. Columns B, Table VI, comprise a summary of the titration of precipitins in each of the sera against each of the soluble substances. The result in each instance is arbitrarily indicated as ++ when a definite precipitin ring was formed between the serum (diluted 1:1) and a 1:1,000,000 or 1:100,000 solution of the soluble substance, and as + when a precipitate formed with a 1:10,000 or 1:1,000 dilution of soluble substance. Comparison of these results

TABLE VI

Comparison of Passive Anaphylaxis with Precipitin Reactions Using Fungus Antisera and Soluble Substances

Soluble substance from organism	<i>M. albicans</i> antiserum		<i>M. psilosis</i> antiserum		<i>M. para- psilosis</i> antiserum		<i>Willia anomala</i> antiserum		<i>Sacchar- omyces cerevisiae</i>	
	A. Passive ana- phylaxis	B. Precipitin titer	A. Passive ana- phylaxis	B. Precipitin titer	A. Passive ana- phylaxis	B. Precipitin titer	A. Passive ana- phylaxis	B. Precipitin titer	A. Passive ana- phylaxis	B. Precipitin titer
1. <i>M. albicans</i>	++	++	++	++	++	+	0	0	+	+
2. <i>M. psilosis</i>	++	++	++	++	+	+	0	0	0	0
3. <i>M. parapsilosis</i>	++	++	0	0	++	++	0	0	0	0
4. <i>Willia anomala</i>	0	0	0	0	0	0	++	++	+	++
5. <i>Saccharomyces cerevisiae</i>	0	0	0	0	+	+	0?	+	++	++

++ = A. 0.1 mg. or less of soluble substance killed, or

B. Precipitin ring with 1:1,000,000 or 1:100,000 dilution of soluble substance.

+ = A. Required more than 0.1 mg. to kill, or

B. Precipitin ring with 1:10,000 or 1:1,000 dilution of soluble substance.

with the anaphylactic response shows a relatively close correspondence between the two phenomena.

DISCUSSION

Although it has not been possible to sensitize actively a limited series of guinea pigs to the soluble polysaccharide-containing fraction of *Monilia psilosis* by repeated injection of the soluble substance, such a hypersensitivity can be demonstrated after repeated injection of the killed organisms. As little as 2 mg. of the soluble substance adminis-

sea Pigs Passively Sensitized with Fungus Antiserum

Intracutaneous injection of rabbit antiserum prepared against

<i>Ionilia paraphilosis</i>	4. <i>Willis anomala</i>	5. <i>Saccharomyces cerevisiae</i>
-+	2.0 mg. -0 / 0.25 mg. H* +	1.5 mg. ++
-+	0.5 mg. -0 / 0.25 mg. H* ++	0.5 mg. -0 / 0.2 mg. H +
++		0.1 mg. -0 / 0.2 mg. H +
-0 / 0.05 mg. H ++		
++	2.0 mg. -0 / 0.25 mg. H* ++	2.5 mg. -0 / 0.2 mg. H +
++	1.0 mg. -0 / 0.1 mg. H +	1.0 mg. -0 / 0.25 mg. H ++
+	0.5 mg. -0 / 0.25 mg. H* +	0.1 mg. -0 / 0.25 mg. H ++
++		0.1 mg. -0 / 0.1 mg. H ++
+		
-0 / 0.05 mg. H +		
++	2.0 mg. -0 / 0.1 mg. H ++	2.0 mg. -0 / 0.2 mg. H +
++	0.5 mg. -0 / 0.15 mg. H* +	0.1 mg. -0 / 0.25 mg. H ++
++		
-0 / 0.05 mg. H ++	0.1 mg. ++	0.5 mg. ++
-0? / 0.05 mg. H +	0.1 mg. ++	0.2 mg. +
-0 / 0.05 mg. H ++	0.05 mg. +	0.1 mg. +
	0.05 mg. +	
	0.03 mg. +	
+	2.0 mg. -0 / 0.15 mg. H +	2.0 mg. ++
++	1.0 mg. ? / 0.1 mg. H -0	1.0 mg. ++
+	1.0 mg. ?	0.25 mg. ++
-0 / 0.03 mg. H ++	0.5 mg. -0 / 0.1 mg. H +	0.1 mg. ++
-0 / 0.05 mg. H +	0.5 mg. -0 / 0.15 mg. H +	0.1 mg. +
		0.05 mg. +.

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tered intravenously precipitates fatal anaphylactic shock. Furthermore, a high degree of hypersensitivity to the polysaccharide fraction of each of the five yeast-like fungi studied can be readily induced by passive sensitization with rabbit antiserum. From 0.01 to 0.1 mg. of soluble substance induces fatal anaphylactic shock in such passively sensitized guinea pigs. The fractions are apparently non-toxic to non-sensitized guinea pigs. Even if all of the trace of nitrogen present in the material represented protein, which is improbable in the light of the negative protein tests, the amount of protein so administered would be too small to produce anaphylactic death.

Cross-anaphylactic reactions occur between the several antisera and soluble substances, requiring in some cases a larger dose of heterologous soluble substance to kill than of homologous. In other words, the reactions are not highly specific, which is in contrast to the pneumococci (5). The ability of a given serum to passively sensitize a guinea pig to the several polysaccharide fractions parallels the precipitin content of the serum. There are minor quantitative differences but not sufficiently significant to throw doubt upon the identity of the precipitin and the sensitizing antibody.

Accordingly the method of passive anaphylaxis would appear to be of no additional assistance in distinguishing between the yeast-like fungi. It is no more specific than the precipitin or agglutinin reactions. The method of absorption of agglutinin or of precipitin (1) remains the most highly specific of those used.

SUMMARY

1. The polysaccharide fractions from each of five yeast-like fungi produce rapid, fatal anaphylactic shock in guinea pigs passively sensitized with antiserum from rabbits immunized against the killed organisms.
2. Cross-anaphylactic reactions with heterologous polysaccharide fractions are frequent. They parallel closely the cross-precipitin reactions, thus adding evidence in favor of the identity of precipitin and sensitizing antibody.
3. The polysaccharide fraction from *Monilia psilosis* produces anaphylactic death in guinea pigs actively sensitized with killed homologous organisms, but an attempt to sensitize actively with the polysaccharide fraction was unsuccessful.

Woods (9) on dogs was successful. An inflammatory reaction was observed not only in the previously sensitized eye but in the opposite eye as well. A similar bilateral eye reaction following intravenous injection of horse serum was noted by Riehm (10) in rabbits previously given horse serum into the conjunctival sac or anterior chamber. In their studies of local organ hypersensitiveness Seegal and Seegal (11) injected into the anterior chamber of the rabbit eye such antigens as guinea pig erythrocytes and egg albumen. Intravenous injection of the homologous antigen 13 days later provoked conjunctival and ciliary hyperemia with slight chemosis and moderate lacrimation in the sensitized eye. They were able to elicit the reaction, though less intensely, as long as 8 months after sensitization.

EXPERIMENTAL

The substances heretofore used to sensitize and reactivate the eye have been protein in nature. Similar experiments are reported here, using one of the soluble specific substances or polysaccharides obtainable from microorganisms. Since the uncombined polysaccharides have been found to be non-antigenic, the organism itself was used to sensitize the eye, and the homologous soluble substance was subsequently given intravenously as the reactivator. The organism used was the yeast-like fungus, *Monilia psilosis* Ashford, from which had been prepared a fraction (1) appearing to be essentially a polysaccharide.

A monilia suspension was prepared by washing the organisms obtained from a honey agar (Sabouraud) culture, and heating them 1 hour at 56°C. For some of the injections the suspension was made with heat-killed organisms which had been frozen with carbon dioxide snow and ground until many had become fragmented. The usual strength of the suspension was 10 per cent by volume of moist packed organisms. Under cocain anesthesia a small amount of aqueous humor was removed with a needle and syringe from the anterior chamber of one eye of each of twelve rabbits.¹ Without removing the needle (which had been inserted obliquely through the cornea just anterior to the limbus), a somewhat smaller volume of monilia suspension was injected into the anterior chamber. Control animals were given sterile saline or a 5 per cent saline solution of the monilia soluble substance. For a few days after the injection of the monilia the eye was usually moderately inflamed, and in some cases injected material could be seen in the anterior chamber for as long as 10 days. The reaction was less marked and usually of only 1 day's duration in the rabbits receiving saline or soluble substance.

¹ Dr. A. L. Morgan of the Department of Ophthalmology, Presbyterian Hospital, New York City, very kindly instructed us in the eye technique and assisted with some of the clinical observations.

HYPERSENSITIVENESS TO SOLUBLE SPECIFIC SUBSTANCES FROM YEAST-LIKE FUNGI

II. EYE HYPERSENSITIVITY

By E. MOTT, M.D., AND H. D. KESTEN, M.D.

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York)

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The precipitability by antisera of carbohydrate fractions prepared from several of the yeast-like fungi as described in a previous report from this laboratory (1), and the ability of these fractions to induce anaphylactic death in sensitized animals as reported in the preceding paper (2), are to be looked upon as examples of immune reactions with non-antigenic haptens. As already briefly reported (3), another reaction of the same type consists in eliciting an inflammatory response in a previously locally sensitized eye by subsequent intravenous administration of the carbohydrate fraction derived from the sensitizing organism.

In various studies of sympathetic ophthalmia, attempts have been made to produce an inflammatory reaction in a previously locally sensitized eye, as well as the opposite eye, by systemic administration of the homologous antigen. Küm-mel (4) injected serum or uveal emulsion into the vitreous humor of one eye and subsequently reactivated the eye by subcutaneous or intravenous injection of the same antigen. Fuchs and Meller (5) reported the production of an iritis by intravenous injection of human serum into an animal sensitized intraocularly 35 days previously. The reaction took place, however, in only one animal of the series used. Schoenberg (6) injected human serum into the anterior chamber of rabbits, followed in 2 weeks by intravenous injection of the same antigen. This, as well as a similar experiment using tuberculin, was negative. Von Szily (7), working with Arisawa, injected foreign serum between the layers of the rabbit's cornea. Later, with all inflammation absent, intravenous injection of the same antigen caused an "anaphylactic keratitis" in the sensitized eye. Subsequently however, von Szily (8) injected relatively pure pigment from the uveal tract of cattle into the vitreous humor of rabbits. Intravenous injection of large amounts of the same pigment 3 weeks later was without effect. A similar experiment by

bility of the conjunctival vessels was common, particularly in the albino rabbits. This was presumably due to handling and was ignored. When a positive reaction occurred, it reached a maximum in 5 to 7 hours after the intravenous injection of soluble substance and was characterized by marked hyperemia of the circumcorneal conjunctival vessels and often of the vessels of the iris and nictitating membrane.

It was soon obvious that the usual incubation period of 2 weeks was not sufficiently long for reactivation of the eye to be successful, the first positive eye reaction being noted at the end of 2 months. Tested 4 or 4½ months after the original eye injection, five of the twelve rabbits exhibited a positive reaction in the sensitized eye (Table I). The opposite eye was invariably negative and served as a control. Of these five animals one was positive as well at the end of 2 months and of 4 months. Control animals were negative, as were also those rabbits sensitized with monilia but given monilia bodies intravenously instead of soluble substance. One of the latter exhibited a definite eye reaction 2 weeks later when given soluble substance. Five animals tested at 8½ months were negative, although three of these had yielded good reactions at 4 months. No difference was noted between rabbits sensitized with whole and with ground monilia.

One-fourth of the experimental group and one-third of the control animals were albino. Of probably no significance except as a matter of coincidence was the observation that all of the reactors were pigmented animals. The primary inflammatory reaction, however, was, on the whole, more marked and of longer duration in the albino animals.

DISCUSSION

As already stated, monilia suspension was visible in the anterior chamber after injection, sometimes for as long as 10 days. Although not visible grossly, it is quite possible that antigen remained present locally for some time longer. This probably accounts for the inability to elicit a reaction as early as 2 to 4 weeks after the original injection.

Inasmuch as the polysaccharide fraction used in the work contained, as previously reported (1), a small amount of nitrogen (averaging 0.6 per cent in the samples used), it cannot be stated unqualifiedly that the monilia-sensitized rabbit eye can be reactivated by subsequent intravenous injection of the homologous protein-free polysaccharide. The negative character of protein tests would indicate, however, that very little, if any, of the nitrogen is present as protein. The above results, furthermore, are in harmony with those of Tomcsik (12), Tomcsik and Kurotchkin (13), Lancefield (14), Avery and Tillett (15), and Enders (16), who found that bacterial carbohydrates, nitrogen-free in the case of Types II and III pneumococci, had the property of

Except for a small corneal opacity at the site of injection, with often a conspicuous vessel in the adjacent conjunctiva, and a rare posterior synechia, the eye ultimately returned to normal. At various intervals (2 weeks to 8½ months) after

TABLE I

Reactivation of Rabbit Eye by Intravenous Administration of Monilia Soluble Substance at Various Intervals after Local Sensitization with Suspension of Monilia Organisms

Rabbit No.	Anterior chamber of one eye injected with	Eye reaction 5 to 7 hrs. after 50 mg. soluble substance injected intravenously				
		2 to 4 wks.	2 mos.	4 mos.	4½ mos.	8½ mos.
1	0.05 cc., 20% monilia*	0		+		0(b)
3	0.05 " 5% "	0%	0	0		0
5	0.05 " 10% "	0	0	+		0(b)
24	0.1 " 1% " *	0		+		0
25	0.1 " 0.05% " *	0		0		0
38	0.03 " 10% "	0	0	0	0	
39	0.05 " 10% "			0	0	
40	0.05 " 10% "	0	0	0(b)	0	
41	0.05 " 10% "			0%	+	
42	0.05 " 10% "		+(c)	+	+	
43	0.05 " 10% " *			0(b)	0	
45	0.05 " 10% " *			0%	0	
2	5 mg. soluble substance	0	0(a)			
35	5 " " "	0	0	0	0	
37	4 " " "	0	0	0%	0	
4	0.1 cc. saline	0	0(a)			
34	0.1 " "			0		
36	0.1 " "			0		

* Ground monilia used instead of intact organisms.

§ 1 cc. 10 per cent suspension ground monilia given intravenously instead of soluble substance.

(a) 1 mg. of soluble substance instead of 50 mg.

(b) 10 " " " " " " 50 "

(c) 25 " " " " " " 50 "

the original injection, monilia soluble substance, usually 50 mg. in 5 per cent solution, was injected into an ear vein. A smaller dose was used in a few instances, and in four cases ground monilia organisms were injected. Both eyes of each animal were examined at intervals after the injection. A slight increase in visi-

producing anaphylactic shock in guinea pigs passively sensitized with rabbit immune serums prepared against the corresponding organisms. Analogous results with monilia soluble substance are presented in the preceding paper (2). Of interest also is the work of Julianelle (17) who was unable to demonstrate eye reactions to pneumococcus soluble specific substance applied to the scarified rabbit cornea following intracutaneous sensitization with killed pneumococci. His procedure is the converse in a sense, of that reported above. We are inclined, therefore, to interpret the eye reaction to the monilia polysaccharide as another manifestation of the ability of a bacterial hapten to elicit an immune reaction in a properly sensitized medium, in this case the actively sensitized eye.

SUMMARY

The anterior chamber of the rabbit eye was sensitized by the local injection of heat-killed *Monilia psilosis*. Subsequent intravenous injection of a polysaccharide fraction prepared from the same organism elicited a reaction in the sensitized eye in five of twelve rabbits.

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Birger and Rawitsch-Birger (7) noted that the action of the bacterial toxins on tissues of isolated organs was similar to that of other poisons but that there was a definite delay in its development. The action of diphtheria toxin was less marked at room than at body temperature. Between 19° and 22°C. the spasms induced by tetanus toxin ceased. Friedheim (8) studied the effect of *B. anthracis*, and Tanenberg (9) that of the pyogenic cocci.

Krontowski (10) and his collaborators (11) have contributed further important observations on the deleterious action of diphtheria toxin on the growth and metabolism of the cells in tissue culture.

All this experimentation has been directed to the effect of the toxin on the tissues—the dosage that influenced growth or inhibited metabolism as determined by the consumption of sugar and liberation of lactic acid—together with the neutralizing action of antitoxin under various conditions. Also, the effect on the tissues of different animal species susceptible and insusceptible to the bacterial toxins has been tested in these studies. The experiments have not as yet definitely revealed an adaptive response in the cells of tissue culture to the specific toxins, comparable to that obtained by immunization of the animal. Fischer (12), however, records observations indicating an adaptive action, or tolerance to the presence of a foreign protein, in cultures of chicken fibroblasts.

The changes in the toxin have not been studied nor have the conditions under which the toxin is altered been determined. This study extends our early investigations to include experiments to determine the effect on diphtheria toxin of fetal and adult tissue and of the living, growing tissue culture.

Experiments with Fetal and Adult Cardiac Tissue

The myocardial lesions of diphtheria, which have recently been described by Warthin (13) in considerable detail, suggested the selection of cardiac tissue, and the guinea pig was chosen on account of its well known susceptibility.

A preliminary experiment was done in which 2 M.L.D. of standard diphtheria toxin were exposed to an emulsion of fresh embryonic cardiac tissue in Locke's solution, for neutralization.

The tissue was thrown down by centrifugalization. One 250 gm. guinea pig was inoculated with the top half and a second, of like weight, with the lower half of the contents of the centrifuge tube. The time required to produce death indi-

THE NEUTRALIZATION OR DESTRUCTION OF DIPHThERIA TOXIN BY TISSUE*

By AUGUSTUS WADSWORTH, M.D., AND ELLA N. HOPPE

(From the Division of Laboratories and Research, New York State Department of Health, Branch Laboratory, New York, N. Y.)

(Received for publication, March 21, 1931)

In the present stage of our knowledge the most direct approach to the study of the action of bacterial toxins in the tissues and the reactions of different tissues to their poisons appears to be the determination of the changes that take place when the toxin and the tissue cells are brought into contact under experimental conditions. Earlier studies (1) record the absence of any action between diphtheria toxin and the leucocytes or brain tissue of either the dog or the guinea pig; also the absence of reaction between these leucocytes and tetanus toxin, although the observations of Wassermann and Takaki (2) that brain tissue neutralizes tetanus toxin were confirmed. The development of the technic of tissue culture suggested the resumption of experimental study of this field. Meanwhile the literature has recorded the reports of other observers which intimate a selective action of these and of other bacterial toxins on the cells of tissue cultures.

Cultures of various tissues, chiefly from chicken embryos but also from some of the common laboratory animals, such as the guinea pig, rabbit, rat, and mouse, have been studied since Levaditi (3) noted the insusceptibility to diphtheria toxin of the cultures from the hematopoietic system in the dilutions which he used. This is in conformity with our observations on leucocytes but it would appear from his experiments with cultures of heart tissue that these were affected by diphtheria toxin and that they were protected from this action by the presence of antitoxin.

Similarly, Burrows and Suzuki (4) record the action of diphtheria toxin on the growth and vitality of tissue cultures, confirming the observations of Levaditi, as have several more recent investigators. Mendeléeff (5) tested cultures of heart tissue of the guinea pig. Kimura and Ishii (6) found that fibroblasts were not susceptible to the action of the bacterial toxins.

* Presented at the meeting of the American Association of Pathologists and Bacteriologists, Cleveland, Ohio, April 2, 1931.

cated that the first guinea pig had received considerably more, and the second guinea pig considerably less than 1 M.L.D., as would have been the case if the toxin had remained in the liquid, and the tissue in the bottom of the tube had merely replaced a volume of toxin.

Later tests were devised to give more clear-cut results; mature guinea pig cardiac tissue was included for comparison with the embryonic cardiac tissue, since the amount of actual muscle tissue in the embryonic hearts was unknown. The accompanying Table I records those tests. The potency of diphtheria toxin which had been in contact with such tissue was in no way lessened. Cardiac tissue of guinea pigs had no neutralizing or binding action for diphtheria toxin. In contrast, as appears from the following experiments, the growing cells in tissue cultures possess the power of completely neutralizing, binding, or destroying the diphtheria toxin so that the tissue of normal guinea pigs is wholly protected from the usual effect of the toxin.

Experiments with Embryonic Cardiac Tissue Cultures

Tissue cultures of embryonic guinea pig cardiac muscle were grown *in vitro* according to the method described by Hoppe (14), except that instead of one fragment of tissue being cultured, eight were placed in each slide equidistantly. The area to be covered with growth was about 113 sq. mm. Great care was taken to have the pieces of tissue adhere to the surface of the clotting medium but not to be submerged in it, since it was desirable that the growth should spread over the surface.

When the surface had become almost covered with tissue, which required 2 or 3 days, it was washed with embryo extract which was immediately removed with a capillary pipette, and then the cultures were exposed to 0.1 cc. of a solution of diphtheria toxin so diluted with Locke's solution that the 0.1 cc. contained exactly 1/500 M.L.D. of toxin.

Control preparations of clotted medium were also covered with like doses of toxin solution. Other controls, with and without tissue cultures, were prepared, to which the toxin solution was added after its potency had been destroyed by heating to 100°C. for 3 minutes and cooling. The cultures and the control preparations were all returned to the incubator and left for 48 hours.

White guinea pigs weighing from 250 to 275 gm. were carefully shaved on the ventral surfaces. The toxin solution was completely withdrawn from the growing tissue cultures and the control preparations into 0.5 cc. syringes graduated to tenths of a cubic centimeter and fitted with hypodermic needles, gauge 26. A separate syringe was provided for each preparation. These 0.1 cc. doses were injected intracutaneously into the guinea pigs. The tissue cultures were returned

TABLE I
Effect of Exposure with Embryonic and Mature Cardiac Tissue upon the Potency of Diphtheria Toxin

Tissue emulsion (0.5 cc. used)	Diphtheria toxin dilution	Exposure for neutralization	Preparation of inocula	Method of inoculation	Results
0.1 gm. of embryonic guinea pig cardiac tis- sue in 0.5 cc. Locke's solution	1 M.L.D. of standard diphtheria toxin in 0.5 cc. Locke's solu- tion	3 hrs. at 37.5°C. in the dark	Centrifugalization, 3 min. at low speed. Supernatant fluid drawn into syringe with hypodermic needle. Sediment washed in 0.5 cc. Locke's solution, centrifugalized, and supernatant fluid drawn into same syringe Sediment washed in 0.1 cc. Locke's solution, centrifugalized, superna- tant fluid drawn into unrinsed syringe Sediment suspended in 1 cc. Locke's solution	Subcutaneous	Death in 91 hrs.
				Intracutane- ous	*No skin reaction
				Subcutaneous	No reaction
0.1 gm. of mature guinea pig cardiac tissue in 0.5 cc. Locke's solu- tion	1 M.L.D. of standard diphtheria toxin in 0.5 cc. Locke's solu- tion	4 hrs. at 37.5°C. in the dark	Centrifugalization 3 min. at low speed. Supernatant fluid drawn into syringe with hypodermic needle. Sediment washed in 0.5 cc. Locke's solution, centrifugalized, and supernatant fluid drawn into same syringe Sediment washed in 0.1 cc. Locke's solution, centrifugalized, super- natant fluid drawn into unrinsed syringe	Subcutaneous	Death in 98 hrs.
Control	1 M.L.D. of standard diphtheria toxin in 0.5 cc. Locke's solu- tion		Syringe rinsed in 0.5 cc. Locke's solu- tion	Intracutane- ous	*No skin reaction
				Subcutaneous	Death in 91 hrs.

White guinea pigs, 250 to 255 gm., were used.

* A typical Schick reaction results from 1/500 M.L.D.

the diluted diphtheria toxin required most careful handling at all times in order to avoid deterioration.

SUMMARY AND CONCLUSIONS

As determined by the intracutaneous test in guinea pigs, diphtheria toxin is not altered in the presence of cardiac tissue obtained from the fetal or from the adult heart of the guinea pig.

Tissue cultures were apparently uninjured by the presence of the toxin in the dilutions used in these experiments, and, when washed with embryo extract after removal of the diluted toxin, continued to grow.

Embryonic guinea pig cardiac muscle tissue growing in cultures *in vitro* possesses the power of neutralizing, binding, or destroying diphtheria toxin so that it is no longer toxic for normal guinea pigs.

Such neutralization takes place through the intervention of growing tissue and is a property which is lacking in similar surviving tissue not in a state of cultivation.

Thus, it appears that the living, growing cells of the tissues neutralize or destroy limited quantities of toxin; only when the quantity of toxin exceeds a certain limit is its action injurious.

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to the incubator where they continued growing and pulsating. The guinea pigs were kept under close observation.

Guinea pigs injected with the toxin solution which had been exposed to growing tissue preparations showed no skin reaction. Guinea pigs injected with the toxin solution from control preparations, but without the presence of growing tissue, showed typical skin reactions. Guinea pigs injected with the toxin solution which had had its potency de-

TABLE II

*Effect of Exposure with Growing Tissue upon the Potency of Diphtheria Toxin.
Protocol of a Typical Test*

Toxin			Skin reaction
Amount	Dose in Locke's solution	Exposed for 48 hrs. at 37.5°C.	
1/500 M.L.D. standard diphtheria toxin	0.1	On tissue culture medium without tissue present	Reaction like Schick test
1/500 M.L.D. standard diphtheria toxin	0.1	On tissue culture growing on culture medium	No reaction
1/500 M.L.D. standard diphtheria toxin	0.1	On tissue culture growing on culture medium	No reaction
1/500 M.L.D. standard diphtheria toxin	0.1	Heated 100°C. for 3 min. and cooled. On tissue culture medium without tissue present	No reaction

For this test a normal white guinea pig, 250 gm., was used.

Four inoculations were made: anterior right, anterior left, posterior right, posterior left.

stroyed by heat showed no skin reaction. One guinea pig was used for several intracutaneous injections. Table II is a protocol of a typical test.

The tissue cultures were uninjured by the presence of the diluted toxin, and if they were occasionally washed with embryo extract after the removal of the diluted toxin, they could be kept living and used for repeating the experiment after several days. On the other hand,

EXPERIMENTAL

Rats in the postabsorptive state, weighing 140 to 165 gm., were radiated. They were tied on the back and the body was shielded with 2 mm. lead with the exception of the part to be radiated. A Victor machine with Snook rectification was

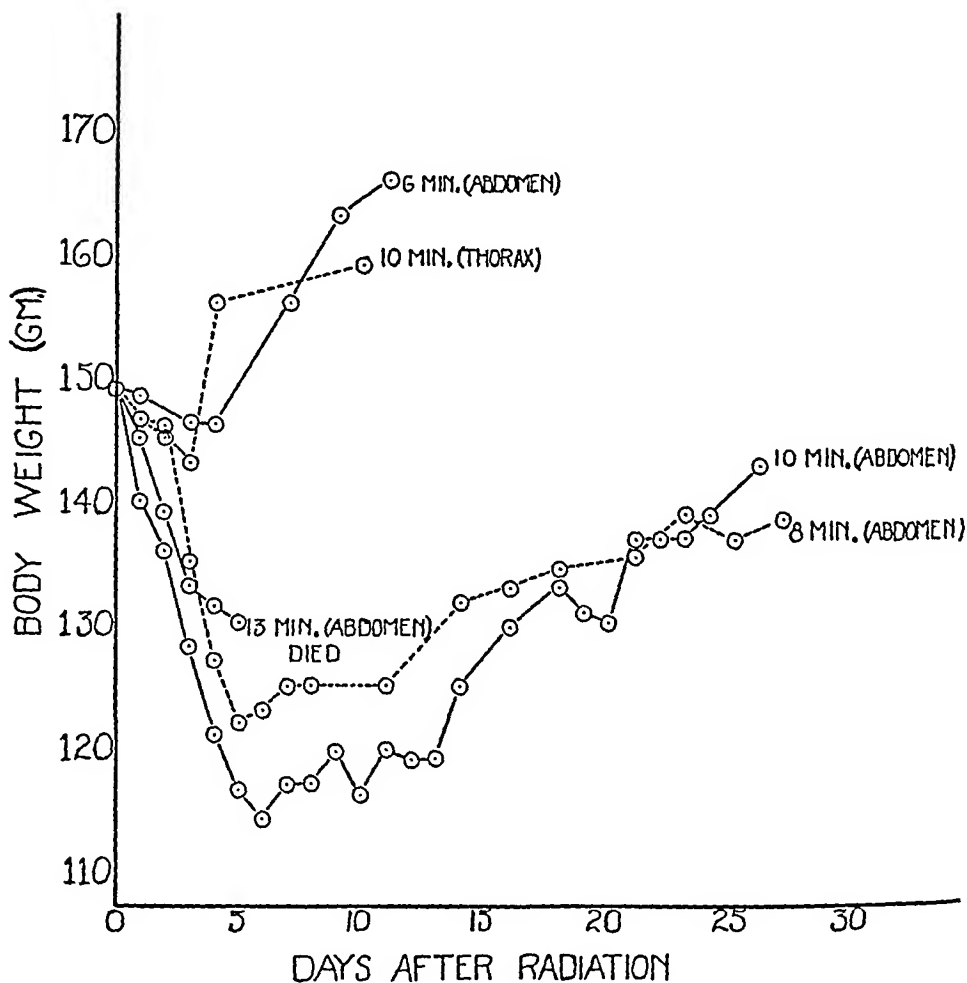


FIG. 1. Body weight of rats following radiation with different doses of x-rays. The conditions of radiation were as follows: 140 k.v.p. 5 ma. 25 cm. s.t.d. 2.5 mm. aluminum filter.

used, equipped with a stabilizer in the filament circuit, thus insuring a very constant x-ray output. An air-cooled Coolidge tube was operated at 5 ma. and 140 k.v.p. By actual ionization measurement the effective wave length of the radiation was 0.3 Å. The animals were placed at a distance of 25 cm. from the target and a filter of 2.5 mm. of aluminum was used.

THE INFLUENCE OF X-RAY LESIONS OF THE INTESTINAL MUCOSA ON ABSORPTION OF GLUCOSE AND OTHER SUGARS

By K. W. BUCHWALD

(From the State Institute for the Study of Malignant Disease, Buffalo)

(Received for publication, March 3, 1931)

Warren and Whipple (1) exposed the abdomen of dogs to large doses of x-rays. Up to the 2nd day after radiation the animals appeared normal; on the 3rd day they became nauseated, refused food and developed an intensive diarrhea. Death generally occurred on the 4th day, preceded by extreme prostration. A histological study revealed that the mucosa of the small intestine from the pylorus to the ileocecal valve was more or less affected. Damage of the nuclei of the crypt cells could be detected a few hours after exposure to radiation. Cell injury was definite after 24 hours and after 48 hours a disintegration set in which coincided with the appearance of clinical symptoms. 72 hours after radiation the destruction was quite extensive and at the time of death practically the whole mucosa had sloughed away. A marked increase in nitrogen excretion in the urine (Hall and Whipple (2)) and a fall in the alkaline reserve of the blood (Denis and Martin (3)) were noted after radiation.

Radiation of the abdomen of laboratory animals other than dogs (cats, rabbits, rats and mice) produced the same effects (4). The onset of diarrhea and of other clinical symptoms was always preceded by a definite latent period during which the well-being of the animals was apparently not disturbed. In rats and mice this latent period was found to be 2 to 3 days. Cori (5) established the minimal lethal x-ray dose for abdominal radiation of the mouse; when more than three times the lethal dose was administered, the latent period was not appreciably shortened nor was the occurrence of death accelerated. The intestinal mucosa of mice proved to be about three times more sensitive to x-rays than the skin.

It has seemed of interest to investigate whether the lesions which can be demonstrated histologically during the period prior to clinical symptoms after x-raying, are accompanied by a disturbance in physiological activity. In order to test the function of the epithelial cells, the rate of absorption of glucose and other sugars was determined in rats 20 and 40 hours after applying a sublethal dose of x-rays over the entire abdomen. Another group of rats received the same dose of x-rays over the thorax and served as control.

rats was there any skin reaction or falling out of hair. As a control a rat was given a radiation of 10 minutes over the thorax; its body weight dropped only 0.4 per cent.

The rate of absorption of sugar from the intestine of rats treated with x-rays was determined by means of a method described previously (6). On each experimental day 2.4 cc. of the same sugar solution was fed by stomach tube to a rat radiated over the thorax and to one radiated over the abdomen. The two animals were of the same sex

TABLE II

Absorption of 40 Per Cent Glucose Solution

40 hours after radiation and 24 hours after last feeding.

Thorax radiated				Glucose fed	Abdomen radiated			
Glucose absorbed per 100 gm. rat per hr.	Glucose absorbed in 2 hrs.	Loss of original weight	Body weight		Body weight	Loss of original weight	Glucose absorbed in 2 hrs.	Glucose absorbed per 100 gm. rat per hr.
mg.	mg.	per cent	gm.	mg.	gm.	per cent	mg.	mg.
231	634	7.1	137	906	142	8.0	246	87
182	534	5.1	147	931	149	3.8	181	60
194	560	5.5	144	938	148	7.2	242	81
173	535	4.3	154	963	145	5.5	356	122
211	636	5.9	150	981	146	7.9	353	120
				905	157	6.0	242	77
				905	154	9.1	214	69
				905	157	11.0	238	72
Average. .198 ±16	579	5.5	146	931 (924)*	151	7.3	259	86 ±18

* Average amount of glucose fed to rats radiated over thorax.

and of approximately the same weight. After 2 hours the rats were killed and the entire intestinal tract was analyzed for its sugar content, using Bertrand's method. The amount of sugar absorbed corresponded to the difference between the amount fed and that recovered from the intestinal tract. The glucose and fructose used were Pfanstiehl products, while the mannose was obtained from the Eastman Kodak Company.

Table I shows that 20 hours after x-ray treatment the rats radiated over the thorax had absorbed an average of 206 mg. of glucose, while

As a preliminary step it was necessary to determine the lethal dose of x-rays for the standard conditions described above. A radiation of 16 minutes, applied over the entire abdomen, led to death on the 4th day, the animal having lost 21.5 per cent of its original body weight. Another rat receiving a radiation of 13 minutes duration died on the 5th day, the loss in body weight amounting to 12.7 per cent. Two rats which were given a radiation of 10 and 8 minutes survived, the greatest loss in body weight being 25.3 and 18.0 per cent respectively.

TABLE I

Absorption of 40 Per Cent Glucose Solution

20 hours after radiation and 24 hours after last feeding.

Thorax radiated			Glucose fed	Abdomen radiated		
Glucose absorbed per 100 gm. rat per hr.	Glucose absorbed in 2 hrs.	Body weight		Body weight	Glucose absorbed in 2 hrs.	Glucose absorbed per 100 gm. rat per hr.
mg.	mg.	gm.	mg.	gm.	mg.	mg.
184	583	158	895	158	460	145
194	603	155	915	165	422	128
250	772	154	900	154	633	204
224	760	169	915	168	539	163
167	580	173	930	160	372	116
204	590	144	910	149	560	187
222	640	144	860	143	635	220
Average.. 206 ±22	647	157	903	156	517	166 ±32

A radiation for 6 minutes caused a loss in body weight of only 0.2 per cent (Fig. 1).

In order to ascertain whether 10 minutes radiation was really a sub-lethal dose of x-rays, seven rats were radiated for this length of time. All of them survived, although they showed marked symptoms of x-ray intoxication. 2 or 3 days after radiation the animals became depressed and refused food. On the 4th day diarrhea developed and the body weight dropped markedly. Recovery set in on the 7th or 8th day and the animals slowly regained their original body weight. A composite weight curve for all the rats receiving 10 minutes radiation over the abdomen is shown in Fig. 1. In none of the radiated

40 hours after radiation could not be carried out because the sugar feeding was followed by diarrhea. Not only the rate of absorption of glucose but also that of fructose and mannose was markedly diminished following radiation of the abdomen (Table III). It may be assumed that this general impairment of the absorbing capacity of the intestine is a contributory factor to the intense diarrhea which develops at a later stage of x-ray intoxication.

The normal epithelial cells of the small intestine show a selective action on sugars, absorbing some more rapidly than others (6). It seemed of interest to investigate whether this selectivity was maintained after x-ray damage of the intestinal epithelium. The ratio in the rate of absorption of glucose, fructose and mannose in the control rats in Tables II and III was of the order 100:42:13. This is similar to the ratio of 100:43:19 obtained in a previous investigation (6). 40 hours after abdominal radiation the ratio in the rate of absorption of these three sugars was as 100:60:11. It may be concluded that the epithelial cells maintained their selective action on sugars.

Attention should be called to the fact that the average decrease in body weight in the first 40 hours after x-ray treatment was the same in the rats radiated over the thorax as in those radiated over the abdomen (Tables II and III) and that all animals appeared clinically normal at the time of sugar feeding.

Histological Findings

The author is indebted to Dr. K. Terplan of the Buffalo General Hospital for the following report.

Pieces of duodenum, jejunum and ileum were taken from rats killed 20 and 40 hours after radiation and from untreated rats. All animals had remained without food for 24 hours. The only macroscopically observable change was an increase in the amount of fluid in the intestinal lumen 40 hours after radiation. The microscopical findings were:

20 Hours after Radiation.—The chief pathological changes were to be noted in the crypt cells, consisting of various forms of degeneration of the nuclei. The latter were enlarged, took the stain poorly, the chromatin was granulated and resembled nucleoli. Other cells showed disintegration of the nuclear substance and formation of large vacuoles with inclusion of chromatin fragments. Some epithelial cells in which there occurred a marked mucin formation assumed the typical signet ring shape. Fragments of leucocytes were observed within a few

those radiated over the abdomen had absorbed only 166 mg. per 100 gm. rat per hour, a decrease of 19.4 per cent. 40 hours after radiation of the abdomen the average glucose absorption amounted to 86 mg. per 100 gm. rat per hour, a drop of 56.5 per cent when compared with the average absorption of 198 mg. per 100 gm. rat per hour of the con-

TABLE III

Absorption of 30 Per Cent Fructose Solution

40 hours after radiation and 24 hours after last feeding.

Thorax radiated				Sugar fed	Abdomen radiated			
Sugar absorbed per 100 gm. rat per hr.	Sugar absorbed in 2 hrs.	Loss of original weight	Body weight		Body weight	Loss of original weight	Sugar absorbed in 2 hrs.	Sugar absorbed per 100 gm. rat per hr.
mg.	mg.	per cent	gm.	mg.	gm.	per cent	mg.	mg.
77	223	3.0	144	600	148	2.6	144	48
97	303	5.7	156	660	161	4.1	197	61
73	224	5.6	152	660	141	6.3	137	48
Average..82	250	4.7	152	640	153	4.3	159	52

Absorption of 20 Per Cent Mannose Solution

40 hours after radiation and 24 hours after last feeding.

Thorax radiated				Sugar fed	Abdomen radiated			
Sugar absorbed per 100 gm. rat per hr.	Sugar absorbed in 2 hrs.	Loss of original weight	Body weight		Body weight	Loss of original weight	Sugar absorbed in 2 hrs.	Sugar absorbed per 100 gm. rat per hr.
mg.	mg.	per cent	gm.	mg.	gm.	per cent	mg.	mg.
20	60	4.9	145	460	142	6.5	20	7
27	86	4.9	154	423	150	4.1	30	10
27	63	6.2	135	440	140	3.9	40	13
Average..25	69	5.3	144	441	150	4.8	30	10

trol rats (Table II). In the last three experiments in Table II stomach and intestine were analyzed separately; the latter contained 8, 71 and 47 mg. of sugar respectively. If slow evacuation of the stomach were responsible for the diminished rate of absorption after radiation, the intestine should be free of sugar. Experiments at a later period than

epithelial cells. There was a distinct diminution in the number of mitoses in the basal part of the intestinal glands. The whole gland structure was hazy. An increase in the number of leucocytes occurred in the stroma, some of them showing signs of disintegration. In the lymph follicles, especially those of the ileum, the lymphocytes showed marked nuclear disintegration and giant cells filled with nuclear fragments made their appearance.

40 Hours after Radiation.—The haziness of the structure and the number of leucocytes in the stroma were both increased. Mucin formation was even more marked than after 20 hours. There were again to be noted distinct signs of disintegration of the nuclei and invasion amongst the epithelial cells of leucocytes. More mitoses could be counted than after 20 hours but decidedly less than in the controls; there were also pathological mitoses with disintegration of the chromatin. The degenerative changes in the lymph follicles, which were so outspoken after 20 hours, had disappeared entirely. Summarizing it may be said that the early histological changes in the mucosa of the rat were very similar to those described by Warren and Whipple (1) in the dog, except that these authors did not mention the diminution in the number of mitoses and the disappearance of changes in the lymph follicles after 40 hours.

SUMMARY

A sublethal dose of x-rays was applied over the abdomen of rats and over the thorax for control purposes. 20 and 40 hours after radiation (*i.e.* during the "latent period") the rate of absorption of glucose, fructose and mannose was markedly diminished. Simultaneously definite histological changes were observed in the intestinal mucosa. In spite of the decrease in the absolute amount absorbed, the relative rate of absorption of the three sugars mentioned remained nearly unchanged, indicating that the epithelial cells retained their selective action on sugars.

The author wishes to take this opportunity to thank Dr. C. F. Cori for his helpful advice in carrying out this problem.

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outbreaks, to all the adults in the herd. As a rule the calves are not appreciably affected.

In general the more severely affected animals are dull, refuse food, and at times exhibit signs of abdominal pain. The feces are fluid, in some cases fetid, usually deep brown to greenish black in color, and often contain blood and mucus. The milk yield is greatly diminished. The diarrhea usually runs a relatively short course from a few hours to 4 or 5 days, when the feces become firmer, the general health improves, and the milk yield begins to increase. In certain instances cows apparently recover but when placed on full diet diarrhea recurs. At times the onset is accompanied by a dry cough and excessive salivation. As a rule the respirations are rapid.

Four cases were autopsied. One was from a small herd where two other cows had died of the disease, two others were slaughtered when acutely ill, and the fourth was a cow which had suffered from an acute attack and after apparent recovery again scoured when heavily fed.

Examination of material from these individuals indicated a catarrhal inflammation of the small intestine (jejunum and ileum) inasmuch as the serosal vessels were injected, the intestinal walls edematous, and the mucosa swollen, wrinkled, and congested. The intestinal content was fluid, usually dark brown in color, and admixed with mucus. The liver in three instances was ochre color, dry in consistency, and readily fractured when bent, and the gall bladder was engorged with dark bile. The heart muscle was dry and brownish red in color. In addition the folds of the abomasum were congested and edematous. The mesenteric lymph glands were enlarged, pale, and juicy.

Histologically well marked changes were observed in the sections of the small intestine where a well defined vascular engorgement was noted. The superficial mucosa was degenerated and overlain with mucus and disintegrated cells. The mucosa was infiltrated with round cells and leucocytes, the vessels of the submucosa engorged, and the connective tissue edematous. Sections of the liver in three of the four cases revealed hydropic degeneration of the parenchymatous cells.

Bacteriological Findings from the Spontaneous Cases

Cultures from liquid feces from acute cases in all outbreaks were made on lactose agar plates containing indicator. From the cows in three herds actively motile Gram-negative rods which failed to ferment lactose were obtained, and at times such organisms made up 90 per cent of the organisms on the plates. From other cases in the same herd they could not be cultivated. Later organisms of this type were correlated with *B. coli mutabile* and when fed to healthy calves failed to establish themselves in the intestinal tract.

THE ETIOLOGY OF INFECTIOUS DIARRHEA (WINTER SCOURS) IN CATTLE

By F. S. JONES, V.M.D., AND RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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To judge from information from various sources there exists in certain dairying sections of this country an epidemic form of diarrhea among cows. Such outbreaks are said to occur during the fall and winter months and for this reason the disease is frequently referred to as winter dysentery. Little is known concerning its etiology, and although both Steffen (1) and Marshall (2) recognize it as an infectious disorder, others hold the view that it is a dietary disease without specific cause. In the main the disease is said to be an afebrile one characterized by the passage of dark brown or blackish brown liquid feces, and because of this fact it is known in certain localities as black scours. The mortality varies; Steffen states that the condition is rarely fatal but Marshall reports 16 deaths among 62 affected cows. All agree that the disease seriously affects the milk yield both during and subsequent to the attack.

The Characterization of the Disease

In all we have had access to 5 herds in which a total of 400 cows were affected.¹ In the main there was relatively little fever although in one small group in which three fatalities occurred two had temperatures of 39.6° and 40.2°C. respectively. The onset appears suddenly; usually a few cows begin to scour, then the diarrhea may spread rapidly throughout the particular group or, as we observed in two

¹ We wish to acknowledge our indebtedness to Drs. J. H. Herron of Bordentown, N. J., E. H. Hopper of Ridgewood, N. J., and G. H. Kimnach of Hightstown, N. J., through whose cooperation we were able to visit herds in which the disease existed.

inoculated into the condensation fluid of tubes of slanted agar to which a few drops of horse blood agar had been added. From the first tube two others in series were inoculated. All tubes were then sealed with sealing wax and after suitable incubation the condensation fluid was examined. Frequently the tubes inoculated directly with the suspension developed rapidly growing organisms, but from the secondary tubes what appeared to be pure cultures of tiny motile vibrios were obtained.

The next calf (1655) was fed liquid feces, mixed with salt solution, from three acute cases of diarrhea. The spontaneous disease had attacked about half the herd up to the time of our visit and within the next week practically every animal developed the disease. The feces which we fed to Calf 1655 contained besides *B. coli*, *B. coli mutabile* in considerable numbers. Little of note indicative of enteritis was observed as the result of the artificial infection except that the calf was constipated, the feces clay colored and always contained large masses of clay colored mucus. The animal was slaughtered 12 days after the feces were fed. On autopsy the liver was yellowish brown in color and friable, and on section the color extended throughout. The cut surfaces were granular. The upper and middle portions of the jejunum revealed gross changes similar to those observed in the intestines of Calf 1629. In addition the mucosa of the ileum was swollen and reddened for a distance of 1 m. above the ileocecal valve. Edema and congestion of the leaves of the abomasum were also noted.

Essentially the same histological picture as that encountered in the case of Calf 1629 was noted in the fixed and stained material from Calf 1655. In the latter case the liver revealed distinct changes such as nuclear degeneration of the liver cells and hydropic infiltration of the cytoplasm of such cells, and passive congestion.

Nothing significant developed in the lactose agar plate cultures inoculated with content of the jejunum and ileum. *B. coli* was not found above the middle ileum.

Media inoculated with bits of liver, spleen, and kidney remained sterile. Fragments of the inflamed mucosa of the jejunum were treated in the manner previously described, and when ground, suspended in broth, and inoculated into blood agar, developed after suitable incubation under seal vibrios that resembled those obtained from Calf 1629.

The third calf (1641) when 16 days old was fed feces mixed with salt solution from three acute cases of diarrhea. This material was obtained from another large herd where the disease within the course of 7 days attacked over 160 animals. The scouring was severe in the milch cows but relatively mild in the bulls and young stock. The feces contained no cultivable organism of the colon group other than *B. coli*. Within 24 hours the calf was depressed, and on the 2nd day there was an elevation of temperature (39.5°C.). On the 3rd day the depression was more marked, and the feces were soft, yellow in color, and fetid. The calf's condition improved on the 4th day and it was slaughtered on the 5th day. Autopsy revealed an orange-red liver with rounded borders. There was a well marked patchy inflammation of the mucosa of the small intestine extending

Blood cultures from acute cases failed to develop suggestive organisms. Media inoculated with bits of organs from the slaughtered animals remained sterile, and inoculations from the intestinal content and mucosa of various portions of the small intestine revealed either *B. coli* or mutable colon bacilli in enormous numbers from all portions of the small intestine.

The only evidence indicating that the disease was an infectious one seemed to lie in the fact that it spread from group to group, but this fact might be explained by some toxic substance in the food or drink. It seemed essential to attempt to reproduce the disease, and our first protocols deal with such experiments.

EXPERIMENTAL

A 4 months old calf (1629) was fed with a small quantity of intestinal content obtained from the jejunum and ileum of a spontaneous infection. The spontaneous case originated in a large dairy herd where diarrhea had been prevalent for 6 weeks. This case was one of the last to appear and after apparent recovery again had diarrhea when heavily fed. The calf developed diarrhea 3 days after feeding, the attack lasting 2 days. Diarrhea was noted again on the 7th and 14th days. The animal was slaughtered 16 days after the feces were administered.

At autopsy a well defined inflammation involving the duodenum, jejunum, and upper portion of the ileum was found. The lesions consisted in a well marked engorgement of the vessels of the serosae. The intestinal walls were thickened. The mucosa was swollen and varied from bright red to pink in color. The content was slimy and tinged with bile. Peyer's patches and solitary follicles were frequently swollen and often overlain with deep red swollen mucosa. There was little abnormal observed in the large intestine. The spleen, liver, and kidneys appeared normal.

Histological examination of fixed and stained material revealed well defined changes in the small intestine; frequently the superficial mucosa was degenerated and often overlain with mucus, leucocytes, and necrotic cells. The capillaries of the mucosa were engorged and leucocytes had invaded the mucosa in large numbers. The secreting layer of the mucous glands had been invaded by leucocytes and aggregates of such cells at times plugged the lumen of the gland. The sub-mucosa was congested and edematous.

The inoculation of lactose agar plates with the contents of various segments of the small intestine revealed large numbers of *B. coli* throughout the jejunum and ileum but no other significant organism.

Bits of the mucosa from the jejunum were washed in several changes of sterile salt solution, ground in a glass grinder similar to that recommended by Hagan (3), and the ground material mixed with sterile salt solution. This suspension was

INFECTIOUS DIARRHEA IN CATTLE

TABLE I
The Effect of the Aural Administration of Vibrios

Animal	Age	Fed	Symptoms	Autopsy	Bacteriological findings
Calf 1669	26 days	2 cultures Calf 1629 1 blood agar Calf 1641 1 leptospira Calf 1655	Rise of temperature, diarrhea with mucus for 3 days. Killed on 7th day	Cloudy swelling of liver cells; well marked inflammation of lower duodenum, jejunum, and upper half of ileum	Many cultures of vibrios from two portions of jejunum
Cow 1683	15 mos.	5, 72 hr. blood agar cultures of vibrios from Calf 1669, 3rd transfer	Rise of temperature, suppression of milk. Passage of bloody mucus containing vibrios, mild diarrhea on 5th day. Killed on 6th day	Inflammation of the mucosa of jejunum and ileum	Vibrios mixed with spore-bearing bacilli. Pure cultures not obtained
Calf 1686	15 days	2, 48 hr. blood agar cultures from Calf 1669, 13th transfer	Little general reaction, large quantities of mucopurulent material in feces. Killed on 5th day	Severe inflammation of jejunum and upper half of ileum	Vibrios in large number throughout jejunum and upper ileum
Calf 1630	3½ mos.	3 blood agar cultures from Calf 1655	Diarrhea during first 3 days, rapid recovery. Killed on 8th day	Mild inflammation of jejunum and upper ileum	Vibrios not obtained

throughout the jejunum and into the anterior half of the ileum. Here the mucosa was reddened, swollen, and frequently overlain with tenacious mucus. The folds of the abomasum were swollen and reddened. The intestinal content was yellowish white in color and contained large quantities of mucus.

Histological examination of sections of the liver revealed well marked cloudy swelling of the parenchymatous cells. The histological changes encountered in sections of the intestine were similar to those found in the two other calves.

B. coli as indicated by lactose agar plate cultures was present in large numbers throughout the ileum and in smaller numbers in the jejunum. Pure cultures of actively motile vibrios developed in the tubes inoculated with the washed mucosa of the upper jejunum but from the middle jejunum and ileum only mixed cultures were obtained.

The experiments indicated that when feces or intestinal content from cases of diarrhea were mixed with salt solution and administered to calves by mouth a definite reaction followed. In two instances a slight temperature reaction accompanied by general depression occurred but in all cases a catarrhal enteritis was found at autopsy. In two instances the feces became soft and fetid and in the other case the feces contained large quantities of mucopurulent material. The administration to calves of feces from spontaneous cases resulted then in a disease of the small intestine accompanied by a mild general reaction. The disease although less severe simulated the malady encountered in the older cows.

In addition from the experimental cases we had succeeded in obtaining cultures of tiny, actively motile vibrios from involved portions of the intestine which might be of etiological significance. It is of interest to note that we had seen organisms of similar morphology in preparations of mucus obtained from the feces of cows with diarrhea and that they had been found in small numbers in mixed cultures made from bits of this mucus.

To establish the relationship of these vibrios to the disease a series of experiments were undertaken. The results are recorded in Table I.

It will be noted that the cultures when mixed with the feed or suspended in salt solution and administered by mouth were capable of producing in most instances recognizable symptoms. Inflammation of the mucosa of the jejunum and anterior ileum was a constant finding. From two of the experimental calves (1669 and 1686) a large number of pure cultures of vibrios were cultivated. Cultures from

in one of these some of the first cases occurred among cows fed from an old lot of ensilage; nevertheless cows in the same barn fed beet pulp instead of ensilage came down with the disease, and 2 or 3 days later diarrhea appeared among the cows in other barns where there was no question raised as to the quality of the feed. Cows on outlying farms were later affected, where all feed stuffs were of different lots. Evidence in another instance is indicative of the infectious nature of the malady. Here the first cases occurred among the cows in a wing of the main barn, the disease next appearing in the main barn and other large barns connected by runways with it. There had been no change in the character of the feed prior to the outbreak. Direct proof of the infectious nature of the diarrhea is afforded by the findings here recorded when calves were fed suspensions of feces from spontaneous cases. Two of the calves developed diarrhea and the third clinically manifested enteritis by the passage of large quantities of mucus covered feces and mucopurulent material. All three when killed revealed well defined characteristic inflammation of the small intestine. In all three cases vibrios similar in morphology were obtained from the intestinal tract.

In regard to the nature of the etiological agent it may safely be said that the more common types of organisms which would be suspected in such a disease have been ruled out by the methods employed. If organisms of the colon-typhoid group were responsible it is probable that our methods would have demonstrated them, since we readily cultivated *B. coli mutabilis* from the feces or intestinal tract of certain spontaneous cases. These when fed to calves in amounts as high as 45 cc. of 18 hour broth cultures failed to produce disturbances or, as far as we could tell, to establish themselves in the intestinal tract.

The only organisms encountered which appeared to offer etiologic possibilities were the vibrios isolated from the inflamed intestines of calves which developed enteritis as the result of the ingestion of feces from spontaneous cases. Such organisms had been seen in preparations of mucus obtained from spontaneous cases and in one instance vibrios had been found in mixed cultures made from mucus obtained from the feces. Three calves when fed these cultures developed diarrhea or other evidences of enteritis. A young cow fed a single strain recently isolated from one of the experimental calves developed diar-

the young cow (1683) also contained vibrios but in every instance mixed with rapidly growing bacilli from which they could not be freed. The other calf (1630) developed a mild diarrhea and at autopsy the lesions although not pronounced could be recognized but the organism could not be cultivated.

The only other organisms which might be regarded as of etiologic significance were the mutable colon bacilli. In certain cases they comprised over 90 per cent of the organisms present in the plate cultures made from feces or intestinal contents but in many cases they were not found. Young broth cultures in amounts of 35 and 40 cc. were administered to calves but as far as we could determine they failed to establish themselves in the intestinal tract. In addition the feces fed to Calf 1641 contained mutable colon bacilli in large numbers but we failed to find them in the fecal cultures or in the intestinal tract at autopsy.

DISCUSSION

Our studies indicate that there exists an infectious disease of cows characterized by severe diarrhea accompanied by marked suppression of milk secretion. In general the same type of symptoms were noted among animals in widely separated herds, and when it was possible to obtain material at autopsy the same lesions were found. Mention has been made of the observations of Steffen and Marshall who characterize the disease with which they dealt as an epizootic dysentery. Our experience indicates, to judge from the natural and experimentally produced disease, that the inflammation has a different distribution from that encountered in human dysentery. The small intestine, particularly the jejunum and upper ileum, seem to be the principal locus.

That the condition is an infectious one there can be little doubt. However, for the purpose of discussion, attention must be given to dietary factors. As a rule the first portion of the ration to be looked at askance is the ensilage. In the first outbreak we encountered the sick received the same ensilage as the older cows in which there was no diarrhea. In another no ensilage had been fed, the animals receiving only the food to which they were accustomed. Two other outbreaks afford strong arguments that the disease is infectious. It is true that

rhea with blood stained mucus in the stools, accompanied by almost complete suppression of milk secretion. All four cases had on autopsy an enteritis similar to that encountered in the spontaneous disease and in the calves fed feces from spontaneous cases. From the intestines of two calves fed these cultures vibrios were recovered in pure culture. From the cow vibrios were obtained in mixed culture only, and from the third calf the tubes inoculated with suspension of inflamed intestinal mucosa remained sterile.

SUMMARY

A disease of cows manifested by severe diarrhea has been described. The condition is characterized by the frequent passage of dark brown or black feces, often containing mucus and blood. The principal lesions are catarrhal inflammation of the small intestine and liver degeneration.

By feeding feces from spontaneous cases to calves a similar but milder disease characterized by the same type of enteritis was produced. Vibrios were cultivated from the inflamed intestinal tract of such experimentally induced cases. Pure cultures of the vibrios when fed to other calves, in certain instances, produced diarrhea and a well marked enteritis similar to that observed in both the spontaneous disease and in calves following the feeding of feces from naturally affected cows. Vibrios were recovered from the inflamed small intestine of three out of four animals fed such cultures.

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largely responsible for the slaughter or death of many calves. We have tabulated the more important pathological and bacteriological findings of a number of cases.

TABLE I
The Distribution of Intestinal Lesions and Bacteriological Findings in the Spontaneous Disease

No. of calf	Age	Distribution of lesions	Bacteriological findings	
			Vibrios	The distribution of <i>B. coli</i> as determined by lactose agar plates
	days			
1652	18	Inflammation of small intestine	Pure culture from lower jejunum and upper ileum	
1682	57	Patchy inflammation of jejunum and upper ileum	Mixed culture of vibrios from jejunum	<i>B. coli</i> throughout jejunum and ileum
1699	50	Inflammation most marked in middle jejunum and upper ileum	Vibrios in pure culture from middle jejunum and upper ileum	<i>B. coli</i> and streptococci from middle jejunum and ileum
1700	28	Lower half of jejunum and upper half of ileum	Vibrios in pure culture from the middle jejunum	<i>B. coli</i> not found in jejunum
1728	18	Inflammation throughout jejunum. Severe inflammation in ileum extending close to ileocecal valve	Pure culture from middle jejunum. Vibrios mixed with <i>B. coli</i> in ileum	Middle jejunum sterile. <i>B. coli</i> present in middle ileum
1731	68	Inflammation throughout small intestine	Vibrios not obtained from intestine. All tubes inoculated with bits of liver developed vibrios	<i>B. coli</i> throughout ileum
1745	51	Patchy inflammation of jejunum. Severe inflammation throughout ileum	Vibrios in pure culture from lower ileum	<i>B. coli</i> as far as the middle ileum
1748	56	Inflammation of the abomasum and small intestine	Pure cultures of vibrios from duodenum and mixed from stomach and remainder of small intestine	<i>B. coli</i> in liver and small intestine below the middle jejunum

From Table I it can be said that most of our material was obtained from calves between 18 and 68 days old, but in all probability the ani-

VIBRIONIC ENTERITIS IN CALVES

By F. S. JONES, V.M.D., AND RALPH B. LITTLE, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

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In a previous paper (1) we called attention to a disease of adult cows characterized by profuse diarrhea from which by indirect means we obtained vibrios and these when fed to healthy calves produced a well defined enteritis. It seemed likely that organisms of this class might be responsible for certain forms of enteritis in calves, especially inasmuch as various types of intestinal disease of obscure etiology are known to exist. That vibrios can be the cause of a more or less chronic intestinal disease is brought out in the present paper.

The Disease as Encountered among Calves

During the latter months of 1929 and 1930 many of the older calves in a large dairy herd were affected with a complication of diseases. Through the kindness of Dr. Theobald Smith who studied the respiratory aspect of the problem, we obtained several gastrointestinal tracts of calves suffering with pneumonia but later many cases were encountered where the cause of death or general debility was directly correlated with intestinal disease.

As a rule calves over 2 weeks old were affected. The unthrifty appearance of such animals attracted attention. The tail and buttocks were often covered with feces and the abdomen distended. The feces were soft or gummy in consistency and varied from clay colored to dark brown in color. They frequently contained blood and mucus and when suspended in water were noted to contain irregular, firm, sticky masses, and these when crushed were found on microscopic examination to contain large numbers of leucocytes often embedded in mucus. As a rule the disease as we have encountered it is more or less chronic but a few cases dying after a short illness have been seen. During the later portion of 1929 and the early months of 1930 only a few sporadic cases appeared but with the onset of colder weather a well marked outbreak occurred, and the disease has been

VIBRIONIC ENTERITIS IN CALVES

present in the content in large numbers throughout the ileum and in the lower jejunum; above this region it was present in smaller numbers.

10 days after exposure the feces of Calf 1709 were soft and contained blood. The calf had fever. It was slaughtered 21 days after the experiment started. A moderately severe enteritis which began in the upper portion of the jejunum and extended practically throughout this region of the bowel was encountered. A more severe inflammation was observed in the lower portion of the ileum. Vibrios were overgrown with the ordinary intestinal forms. Cultures from the cultures were overgrown with the ordinary intestinal forms. Lactose plate cultures revealed *B. coli* in large numbers throughout the jejunum and ileum.

Calf 1710 revealed symptoms of gastric disorder and scoured on one occasion. When slaughtered on the 14th day it revealed a mild patchy congestion of the mucosa of the upper jejunum and ileum. Vibrios were not found in the cultures from the intestinal tract and liver. *B. coli* was present in the content in large numbers throughout the small intestine.

The experiment indicates that infection takes place relatively early in life and that in the main the disease encountered in the herd is a relatively chronic one, but the location and general type of reaction encountered in the acute disease is identical with that manifested in cases of longer standing. Furthermore the negative results obtained by the examination of two calves originating from the same source and born about the same time as the calves used in the experiment strengthen the argument that infection is acquired from the spontaneous infection in the older calves and that the disease may be maintained by the introduction of susceptible individuals.

Inasmuch as the indications pointed to the vibrios as the etiological agent it seemed desirable to test their pathogenicity on calves and the following experiment covers this phase of the problem.

Experiment 2.—Calf 1714 when 16 days old was fed three 48 hour blood agar cultures of vibrios obtained from the middle jejunum of Calf 1707. The cultures had been under cultivation for 3 months and during this period had been transferred eleven times. Symptoms indicative of enteritis, other than a slight general depression 2 days subsequent to feeding the culture, were not noted. When the calf was slaughtered 7 days after artificial infection a mild gastrointestinal catarrh was found. The principal involvement was in the upper and middle jejunum and irregularly scattered areas in the ileum where the mucosa was swollen and congested. Vibrios were obtained from the upper jejunum but the primary cultures from the middle jejunum and ileum were overgrown with *B. coli* and vibrios were not obtained from these regions. The liver was sterile.

mals are infected relatively early in life. Mention has been made of the unthrifty appearance of the calves frequently accompanied by chronic diarrhea. Autopsy reveals well marked intestinal lesions.

The folds of the gastric mucosa (abomasum) are often reddened and swollen. The small intestine, from the duodenum to the ileum, reveals well marked changes. The vessels of the serosae are injected and the intestinal walls appreciably thickened. The mucosa is reddened, the folds enlarged, and the solitary follicles swollen and overlain with reddened mucosa. The content is rich in mucus, desquamated epithelial cells, and leucocytes. The liver when involved is yellowish brown in color and fractures easily. The gall bladder is usually well filled with normal appearing bile. The mesenteric lymph glands are pale and enlarged. Vibrios are at times readily demonstrated in properly prepared films from involved portions of the intestine. In other cases they are not readily found. Histologically the intestinal lesions consist of superficial degeneration of the mucosa, engorgement of the vessels of the mucosa, and infiltration of the cores of the villi and mucous layer with round cells and leucocytes. Leucocytic infiltrations into the lumen of the mucous glands are not uncommon, and edema of the lymphoid structure of the solitary follicles with leucocytic infiltration, congestion, and edema of the submucosa is usually found. At times hyperplasia of the endothelial elements of the submucosa has been encountered. The liver cells may be degenerated and infiltrated with fat.

Since most of our cases were regarded as of some duration, it seemed desirable to endeavor to ascertain the nature of the acute disease as it occurred spontaneously. With this in view young vigorous calves were mingled with those presenting chronic symptoms and later slaughtered at an abattoir. The more important details are given in Experiment 1.

Experiment 1.—Three calves ranging in age from 6 to 8 days were housed with a 2 months old calf which presented symptoms of chronic enteritis. Vibrios had been obtained from the inflamed intestines of older calves kept in the same pen. Two younger calves from the same source as those exposed to natural infection were slaughtered at the time the other three were exposed. Their intestinal tracts appeared normal and vibrios were not found. The calves were under observation for periods of 14, 16, and 21 days respectively.

Calf 1707 had fever and diarrhea 10 days after exposure and the feces contained blood. Diarrhea persisted until the 14th day when the calf was slaughtered. Characteristic severe inflammation of the upper and middle jejunum and the lower portion of the ileum was found. Many apparently pure cultures of vibrios were obtained from the washed and ground mucosa from inflamed regions. In addition pure cultures of vibrios were also obtained from the liver. *B. coli* was

nounced as the disease becomes more chronic. That such animals may become a ready prey to other infections must be recognized. Our first cases seemed to be in general of this class and it was assumed that the intestinal disease had been superimposed on other conditions. The experiment in which young vigorous calves were exposed to older calves regarded as chronic cases of enteritis indicated that such infections may be acquired relatively early and may for a time be overlooked. A further argument that the disease is an independent one, and as such is not dependent on a preexisting one, is that calves may actually die as the result of such infection. Under certain conditions when the malady becomes chronic the clinical effects become so pronounced that slaughter is advisable. In one large herd many calves between the ages of 1 and 2 months have recently been slaughtered because of this disease.

The distribution of the lesions is characteristic. The upper half of the jejunum seems to be the most frequent locus, but inflammation of the mucosa of the abomasum is not uncommon and inflamed patches often of considerable size are frequently encountered in the upper, middle, and lower ileum. The process is one of congestion, loss of superficial mucosa, infiltration of the mucosa with leucocytes and round cells, and edema and congestion of the submucosa. The exudate overlying the inflamed mucosa and appearing in the feces is mucopurulent.

The etiology seems clear; the vibrios which were cultivated from the more chronic spontaneous disease, and from the young calves contracting the infection in a natural manner, all appeared alike in morphology and were all actively motile. In addition they all possessed certain well defined nutritive requirements. At times they could be readily demonstrated in films of the inflamed mucosa and were frequently found in many cultures from various portions of the involved intestine. However, in cases of long standing they are not easily obtained, since *B. coli* and other intestinal forms thrive readily in the content and serve to suppress the growth of the delicate vibrios in the test tube. In three instances they were cultivated from the liver. Experimental proof exists that vibrios that have been under cultivation for considerable periods still possess the property of passing from the mouth through the stomach and localizing in the small intestine and there inciting a process resembling that found in the spontaneous infection.

On the whole the reaction was mild. The intestinal process seemed to be regressing but the organism was recovered. It seemed possible that the culture might have lost some of its virulence on cultivation, so that it was decided to test the pathogenicity of a freshly obtained strain and for this purpose the vibrios obtained from Calf 1714 were chosen.

Calf 1721 when 18 days old was fed six 72 hour blood agar cultures of vibrios obtained from the jejunum of Calf 1714. The cultures were of the third transfer and the strain had been under cultivation for 3 weeks. There was an increase in bodily temperature for the first 2 days following artificial infection and during this time the feces became dark, soft, and fetid. These were the only clinical manifestations noted except that the feces throughout the observation always contained large quantities of thick yellow mucus. In certain instances this material made up about half the stool. Microscopically it was composed of thick mucus loaded with leucocytes and epithelial cells. On the 5th day the feces became much softer and the animal was slaughtered.

In contrast to the ill defined clinical manifestations there was a severe inflammation of the small intestine which began at the pylorus and extended throughout the duodenum, jejunum, and the anterior half of the ileum. The intestinal walls were thickened, the vessels of the serosae injected, and the mucosa swollen, wrinkled, and bright red in color. The content was slimy and below the middle jejunum bile-stained. The liver appeared normal.

Many cultures of vibrios were obtained from the washed mucosa of the upper and middle jejunum and from the middle ileum but below this point the tubes were overgrown with *B. coli*. The tubes inoculated with bits of liver remained sterile. Lactose agar plate cultures revealed small numbers of *B. coli* in the content of the middle ileum and large numbers below this point. No *B. coli* and relatively few other colonies developed on the plates filmed with content from the duodenum and jejunum.

Both calves fed cultures of the vibrios manifested relatively slight clinical manifestations, but the organisms were nevertheless recovered from inflamed portions of the bowel. The inflammatory process in both instances resembled that found in the spontaneous disease and those cases which contracted the natural infection under experimental conditions.

DISCUSSION

A disease affecting calves from 18 to 68 days old has been described. In general it may be said that the clinical picture, other than a general condition of malnutrition and unthriftiness often accompanied by attacks of diarrhea and an irregular, moderate increase in bodily temperature, is confusing. Our data indicate that infection may take place relatively early, but the animals manifest at first only slight clinical manifestations, the symptomatology becoming more pro-

SUMMARY

An intestinal disorder of calves has been described. The clinical manifestations are usually observed in calves 2 or more weeks old. Our experiments indicate that infection may take place relatively early in life and may for a time produce only a mild reaction, but as the disease becomes more chronic the clinical manifestations become more pronounced. The anterior portion of the jejunum is the primary locus of infection but in more chronic cases practically the whole small intestine may be involved. Vibrios were cultivated from the inflamed intestinal mucosa in both the acute and more chronic spontaneous cases. Vibrios were also obtained from the acutely involved intestine of young calves experimentally exposed to natural infection. On three occasions similar vibrios were found in cultures from the liver. When a single strain of vibrios which had been under cultivation for 3 months was fed to a young calf subclinical infection was produced and the organism recovered. This strain after three passages on media when fed to a calf produced a severe inflammation of the jejunum and ileum and from these areas the organism was recovered.

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1745, and 1748, all obtained from spontaneous enteritis of calves (13). The fourth series includes Cultures 1707, 1709, 1714, and 1721 from enteric cases in calves experimentally produced, and finally Cultures 1208, 1522, 1524, and 1679 kindly furnished to us by Dr. Smith. *Vibrio fetus* 1660 was also furnished by Dr. Smith.

Method of Obtaining Vibrios from the Intestinal Tract

Two methods have been employed throughout the work. The less reliable may be said to consist in the inoculation of the water of condensation of veal infusion agar, to which five drops of defibrinated horse blood have been added, directly with the intestinal content of inflamed segments of the intestine. Immediate inoculations from the primary culture to secondary and tertiary tubes of the same media with the sterile loop will at times give pure cultures in one tube but overgrowth of intestinal bacteria is apt to occur and vibrios fail to develop.

A more complicated procedure but one on the whole more satisfactory is the following:

The seared serosa is cut, the intestinal tube flattened, and the content gently brushed from the inflamed mucosa. Four or five bits of the superficial mucosa, 1 to 2 mm. in diameter, are removed and washed in five or more changes of sterile salt solution. The mucosa is then ground in a Hagan (14) grinder, a little broth added, and the suspension inoculated by means of a loop into the condensation fluid of blood agar. It is customary to set up three series of two tubes. The first tube of each pair receives 1, 2, and 3 loopfuls of suspension, and after flaming the loop the secondary tubes are inoculated with 1, 2, and 3 loopfuls of the condensation fluid from the primary cultures. All tubes are then sealed with sealing wax and incubated. After 3 or 4 days tubes which show relatively little growth are examined microscopically. Even in spite of prolonged washing in many instances *B. coli*, streptococci, etc., may develop in the primary inoculations, but the secondary tubes are apt to contain only vibrios. In some cases vibrios will be found only in the first tube and then mixed with other organisms from which they can with difficulty be separated. In other instances only vibrios develop in the first of the series and the secondary tubes may or may not contain them.

In early cultures relatively little is manifested in the gross in the blood agar tubes. The condensation fluid is slightly turbid within 4 or 5 days and the blood's buffy coat is thickened. Later delicate lines at the border of the agar become visible and after several transfers

VIBRIOS (VIBRIO JEJUNI, N.SP.) ASSOCIATED WITH INTESTINAL DISORDERS OF COWS AND CALVES

BY F. S. JONES, V.M.D., MARION ORCUTT, AND RALPH B. LITTLE, V.M.D.
(From the Department of Animal Pathology of The Rockefeller Institute for Medical
Research, Princeton, N. J.)

PLATE 31

(Received for publication, March 12, 1931)

Although many vibrios were described following the discovery of the cholera organism relatively few have been shown to be pathogenic for man or animals.

Gamaléia (1) obtained vibrios (*V. metchnikovi*) from the blood and intestines of fowls suffering from enteritis. MacFadyean and Stockman (2) succeeded in cultivating vibrios from abortion disease in sheep and succeeded in reproducing the disease by injection of pure cultures into pregnant ewes. Since then such organisms have been obtained from sheep by Carpenter (3), Welch and Marsh (4), and Graham and Throp (5). Theobald Smith (6) isolated from the diseased placentas and aborted fetuses of cows vibrios (*Vibrio fetus*) which on injection into pregnant cows produced placental disease and abortion. In a series of papers (7, 8, 9) the disease and the etiological agent were discussed. A similar organism had been recognized by McFadyean and Stockman (10) and has subsequently been found to affect cattle in various parts of the world.

In addition to the disease-producing type of vibrios, Smith and Orcutt (11) reported vibrios obtained from the livers of young calves which resembled *V. fetus* in morphological and cultural characters but failed to agglutinate with sera specific for *V. fetus*.

The Source of the Cultures

The vibrios in this study were obtained from several sources but all were of bovine origin. The first three, 1629, 1641, and 1655, presumably originated from cases of infectious diarrhea (12) experimentally reproduced by feeding calves feces from spontaneous cases. The second pair comprises Vibrios 1669 and 1686 which were cultivated from the intestinal tracts of calves fed Cultures 1629, 1641, and 1655. The third group is composed of Vibrios 1652, 1699, 1700, 1728, 1731,

for 6 days but when the feces became thoroughly dried cultures could no longer be obtained. Distilled water is not especially toxic since actively motile forms may survive for 24 hours. The vibrios resist the soluble action of bile.

Pathogenicity for Laboratory Animals

In accompanying papers we have described (12, 13) our experiments dealing with the pathogenic properties of these organisms for cows and calves. It can be said that for laboratory animals the organisms possess little pathogenicity by the usual standards. Guinea pigs and white rats are refractory when injected intraperitoneally. White mice are more susceptible since two of the strains (1655 and 1686) when injected in small quantities intraperitoneally produced multiple necrotic foci of the liver from which the vibrios were cultivated. Other cultures failed to do so.

Strains 1629 and 1641 possessed no pathogenicity for rabbits, but others (1669, 1686, 1731) when freshly obtained and injected intravenously produced well marked febrile reactions and during this phase vibrios were readily cultivated from the peripheral blood and organs. After the fever declined there was established a catarrhal inflammation of the small intestine, at times accompanied by diarrhea. The vibrios were recovered from the inflamed intestinal mucosa. At this stage the blood and organs were sterile.

Feeding culture to rats resulted negatively. In one mouse of ten fed various cultures vibrios were obtained from the small intestine. Vibrios fed to unprepared rabbits fail to establish themselves in the intestinal tract but when 0.5 gm. of sodium bicarbonate is administered a brief interval before the culture is introduced into the stomach, the vibrios reach the small intestine where they produce catarrhal inflammation and can be recovered after 5 days.

The Immunological Relationship of Various Strains as Judged by Agglutination

It was decided to correlate by means of agglutinin the vibrios obtained from epidemic diarrhea in cows and enteritis in calves with those obtained by Dr. Smith, and with this in view a number of rabbits were immunized with living cultures.

the border lines become well defined and a delicate film may spread from the condensation fluid to the lower slant. After prolonged cultivation many strains grow readily on the surface of the slant. From blood agar, cultures may be established in plain agar or in leptospira medium to which a little fresh kidney has been added. After prolonged cultivation on plain agar growth may even be obtained in broth where a heavy tenacious mass is formed on the bottom of the tube.

Certain strains grow well from the start, others adapt themselves slowly. In serum agar shake cultures the growth is on the surface or in a narrow zone just beneath the surface. In leptospira medium the heaviest growth is just beneath the surface (Fig. 1). From these facts it may be inferred that the organisms are not anaerobes.

When some of the condensation fluid of young blood agar is examined by means of the hanging drop actively motile elements are readily detected but it is difficult to ascertain their morphology because of their extremely rapid movement. Three types are frequently visible in the same culture. Short, slightly convoluted forms (Figs. 2 and 3) are the most active, and in suitable preparations one or two flagella situated at the poles may be demonstrated (Figs. 3 and 4). Forms with two or more complete coils as illustrated in Figs. 5 and 6 move more slowly and revolve about the long axis. The extremely long type as encountered in Fig. 7 is rarely motile although the body appears to sway. As the cultures become older clumps occur and cellular fragmentation or granule formation is the rule. In Fig. 5 the edge of a clump of granules resulting from vibronic degeneration is illustrated, and several reveal early degenerative changes, and in Fig. 7 an atypical granule formation is visible.

The cultures stain well with ordinary stains after a longer time than is usually required. In films prepared from tissues only prolonged staining seems to color them but in fresh, unstained preparations of tissue and exudate they may be readily recognized. They are Gram-negative. The optimum reaction for growth is about pH 7.6. The organisms fail to grow in either slightly acid or definitely alkaline media. Cultures soon die when dried in the air and a temperature of 55°C. for 5 minutes kills them. When autoclaved moist cow feces were inoculated with vibrios and stored in the room the vibrios lived

TABLE I
The Agglutination Affinity of Clinical Vibrios

No. of culture	Source	Antiserum Culture 1629								Antiserum Culture 1691							
		Serum dilutions								Serum dilutions							
		1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
1629	Presumably originating from diarrhea in cows	C	C	C	C	C	+++	++	+	++	+	+	-	-	-	-	-
1641		C	C	C	C	C	-	-	-	C	+	C	-	-	-	-	-
1655		C	C	C	C	C	-	-	-	+++	++	++	-	-	-	-	-
1669	Recovered from calves fed Cultures 1629, 1641, and 1655	-	-	-	-	-	-	-	-	+++	++	++	-	-	-	-	-
1686		-	-	-	-	-	-	-	-	C	C	C	+++	++	-	-	-
1652		-	-	-	-	-	-	-	-	C	C	C	+++	++	-	-	-
1699	Obtained from spontaneous enteritis in calves	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1700		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	++
1728		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	++
1731		+	+	+	±	-	-	-	-	C	C	C	C	+++	++	+	++
1745		-	-	-	-	-	-	-	-	±	±	-	C	+++	++	-	±
1748		+	+	-	-	-	-	-	-	C	C	-	-	-	-	-	-
1707		-	-	-	-	-	-	-	-	++++	++++	++++	+++	+++	++	+	++
Intestine	Recovered from naturally induced and artificially infected cases of enteritis in calves	-	-	-	-	-	-	-	-	+++	+++	+++	+++	+++	++	+	++
1707		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	+++
Liver		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	+++
1709		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	+++
1714		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	+++
1721	Obtained by Dr. Smith from calves	-	-	-	-	-	-	-	-	++++	++++	+++	++	++	++	++	++
1208		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	+++
Liver		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	+++
1522		-	-	-	-	-	-	-	-	C	C	C	C	C	C	C	+++
Liver		+	+	±	±	±	-	-	-	C	C	C	+++	++	±	-	±
1524	Obtained by Dr. Smith from case of vibrionic abortion	-	-	-	-	-	-	-	-	C	C	C	+++	++	++	-	++
Liver		-	-	-	-	-	-	-	-	C	C	C	+++	++	++	-	++
1679		-	-	-	-	-	-	-	-	C	C	C	+++	++	++	-	++
Lung		-	-	-	-	-	-	-	-	C	C	C	+++	++	++	-	++
1660	Vibrio fetus*	±	-	-	-	-	-	-	+++	++	+	-	-	-	-	-	-
Vibrio fetus*		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

* *Vibrio fetus* in homologous serum had the following titer: 1:640 1:1,280 1:2,560 1:5,120
++++ ++ + -

Inasmuch as the cultures grow relatively poorly it was a considerable undertaking to obtain sufficient antigen from plain agar growths for the tests. If, however, to tubes 2.5 cm. in diameter containing 10 to 12 cc. of melted veal infusion agar a fragment of guinea pig or rabbit kidney is added, the tube slanted, and later heavily seeded with young culture and sealed with sealing wax, excellent growth is obtained after 3 or 4 days incubation and suspensions of such growth in NaCl solution afford good antigens.

In Table I the results of the agglutination tests are shown. The same form of arrangement as indicated on page 853 has been followed. The higher serum dilutions in the tests with *Vibrio fetus* antiserum have not been shown since this serum fails to agglutinate the heterologous strains at dilutions higher than those shown.

It is clear from Table I that there exist among the intestinal vibrios at least two well defined immunological groups. Two of the strains presumably originating from cases of infectious diarrhea in adult cows (1629 and 1655) agglutinate well with the same serum and when the serum is absorbed with the heterologous strain the agglutinins are equally removed from both organisms.

The large group, which comprises all but one of the calf enteritis group, one of those presumed to have originated from diarrhea in cows, all the vibrios cultivated from the experimentally induced disease, and the cultures given to us by Dr. Smith, forms what appears to be one immunological group. *Vibrio* 1728 is not agglutinated by any serum.

The serum specific for *Vibrio fetus*-Culture 1660 agglutinates slightly many of the cultures in only the lower dilutions.

Although the members of the large group were strongly agglutinated by both Serums 1641 and 1700 it has been possible to show by absorption that certain vibrios possess a more complex antigen. For instance, if Serum 1641 was absorbed with the homologous strain agglutinin was removed for all strains. If, however, Culture 1700, which was strongly agglutinated by Serum 1641, was used for absorption the agglutinin still remains for the homologous organism and a number of others. This is brought out in Table II.

If Culture 1700 possessed only part of the antigen of strains behaving like 1641, then its antiserum should be completely absorbed by organisms possessing the complete antigen. This proved to be correct. Table III indicates the results of the agglutination tests of Serum 1700

which had been absorbed with Culture 1641. In the experiments the serum was tested at dilutions as great as 1:20,000 but agglutination

TABLE II
The Effect of Absorption of Serum 1641 with Culture 1700

No. of culture	Dilutions of serum							
	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
1641	C	C	C	C	C	C	++++	++
1700	+	—	—	—	—	—	—	—
1208	++	+	±	—	—	—	—	—
1652	++++	C	C	C	C	C	++++	+++
1699	++	+	+	+	+	—	—	—
1707	C	C	C	C	C	++++	++	++
Intestine								
1707	+	+	±	—	—	—	—	—
Liver								
1709	+++	++	++	+	—	—	—	—
1714	C	C	C	C	C	C	++++	+++

TABLE III
The Effect of Absorption of Serum 1700 with Culture 1641

No. of culture	Dilutions of serum				
	1:80	1:160	1:320	1:640	1:1,280
1641	++	+	±	—	—
1700	+++	++	+	±	—
1208	++	+	+	—	—
1652	++	++	+	±	—
1699	++	+	±	±	—
1707	++++	++++	+++	+	—
Intestine					
1707	+++	++	+	—	—
Liver					
1709	++	+	±	—	—
1714	++	+	±	—	—

stopped at the titer of 1:640. The higher dilutions have been omitted from the table.

The experiment was repeated but this time 1641 was absorbed with

ities of Intestinal Vibrios

m Culture 1641					Antiserum Culture 1700							Antiserum Culture <i>Vibrio fetus</i> 1660		
m dilutions					Serum dilutions							Serum dilutions		
1:640	1:1,280	1:2,560	1:5,120	1:10,240	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:80	1:160	1:320
+	±	-	-	-	-	-	-	-	-	-	-	-	-	-
C	C	C	++++	++++	C	C	C	++++	++++	++	+	+++	+	±
+	-	-	-	-	+	+	-	-	-	-	-	+++	+	-
+++	+	±	-	-	C	C	C	C	+	±	-	-	-	-
+++	+	±	-	-	C	C	C	++++	+	+	±	-	-	-
C	C	C	++	+	C	+++	++	++	++	±	-	+++	++	-
C	+++	+	±	-	C	C	C	C	++	+	±	-	-	-
C	+++	±	-	-	C	C	C	C	++	±	-	+	-	-
-	-	-	-	-	C	C	C	++++	++	±	-	+++	+	+
+++	+++	+	-	+	C	C	+	+++	++	+	±	+++	+	+
+++	+++	+++	++	+	+++	+++	+++	++	++	+	-	+++	+	±
+	+	-	-	-	+	+	+	+	+	+	-	+++	+	±
C	C	C	++++	++++	C	++++	++++	++++	++	-	-	+++	+	±
C	C	C	+++	+	C	C	C	++++	++	+	-	+++	+	-
+++	+	±	-	-	+++	+++	+++	+	±	-	-	+	-	-
C	C	C	++++	++++	C	C	+++	+++	++	+	-	+++	+	-
C	C	C	+++	++	C	C	C	++++	++	+	-	+++	+	±
+++	+	±	-	-	C	C	C	C	+++	±	-	+	-	-
+++	++	++	-	-	C	C	C	C	C	++	+	++	+	±
C	C	C	++	+	+++	++	++	+	-	-	-	+++	+	+
+++	+++	+++	++	-	C	C	+++	+++	++	+	±	++	+	±
-	-	-	-	-	+++	+++	+	±	-	-	-	C	C	C

fecal mucus after 36 hours. It should be stated that the cultures recovered from the livers of artificially infected mice, and from the peripheral blood, organs, and small intestines of rabbits were proved by agglutination to be similar to those injected.

The grouping according to agglutination affinities has been of assistance to us in certain respects. When Culture 1707, which had been shown to possess the whole antigen of the larger group, was fed to Calf 1714 it gave rise to definite intestinal disease and the vibrio obtained from the jejunum also possessed the whole antigen. When Culture 1714 was fed to Calf 1721 more severe disease was encountered and *Vibrio* 1721 was shown to possess the complete antigen. Evidently the character is transmitted in spite of considerable variations in the environment. That both types may exist in cultures from different regions in the same animal is also true. The vibrios obtained from the intestine of Calf 1707 possessed the whole antigen while the strain cultivated from the liver failed to possess the complete complex. This has been true in other instances, all the vibrios from the liver studied have failed to contain the complete antigenic complex. In regard to the relation of the vibrios originating in the intestinal tract to *Vibrio fetus* all that can be said is that they failed to show the same agglutination affinities as the culture of *Vibrio fetus* employed.

SUMMARY

A number of vibrios obtained from the small intestines of calves fed feces from spontaneous diarrhea in cows, natural intestinal disorders of calves, experimentally induced infections of calves, and cultures obtained from Dr. Theobald Smith have been studied. From the close morphological resemblance, similarities in motility, position and number of flagella, tinctorial properties, and the tendency to fragmentation in older cultures, as well as the narrow nutritive requirements, we are led to regard them as a closely allied group and we propose the name *Vibrio jejuni*.

Immunologically as judged by agglutination the organisms have been divided into two groups, the smaller representing two strains originating from diarrhea in cows and the larger comprising one from this source and many from the calf disease. The larger group can be

Culture 1707 which, from the data in Tables II and III, was considered to comprise the complete antigen. The results indicated that Culture 1707 contained the complete antigen since the titer of the serum for all vibrios was greatly reduced.

It is true that the vibrios with the exception of one strain fall into two well defined groups, the smaller comprising only two strains which both originated from epidemic diarrhea in cows, the larger embracing the vibrios cultivated from the inflamed intestinal tracts or livers of calves. The latter group may be divided on the basis of agglutinin absorption into two types, one possessing a complete antigenic character and the other possessing only a portion of the antigen. It is also true that none of the vibrios are antigenically similar to the culture of *Vibrio fetus*.

DISCUSSION

The vibrios while presenting certain slight morphological differences, such as the length, the number of coils, and to some extent the depth of coils, nevertheless resemble each other sufficiently to be regarded as a closely related group. Since their first locus in the intestinal tract as judged by their presence in lesions encountered in acute infections is the jejunum, the name *Vibrio jejuni* is proposed.

Their pathogenic properties for cows and calves have been discussed in earlier papers. In the main the disease induced resembles to a certain extent human cholera and the vibrios from the bovine disease often resemble in young cultures the human organism. Both maladies are infections of the small intestine and both are characterized by excessive mucous secretion. However the bovine vibrios differ markedly from the comma vibrio in cultural characters. The bovine group are more difficult to grow, fail to liquefy gelatin or blood serum, and will not survive on strongly alkaline media. Thus far they have not been shown by means of acid or gas production to utilize carbohydrate. Similarities in pathogenic properties for rabbits exist, both organisms when introduced into the circulation produce fever and penetrate the intestinal wall. In both cases it is necessary to neutralize the acidity of the stomach to infect rabbits by mouth. No such procedure is necessary in infection experiments with the cow or the calf since the vibrios readily pass the stomach and frequently are observed in the

subdivided by means of agglutination absorption into cultures which do not contain the complete antigenic complex and others which do so.

Certain freshly isolated vibrios when injected into rabbits incite definite reactions terminating in a localization in the small intestine accompanied by catarrhal inflammation.

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EXPLANATION OF PLATE 31

FIG. 1. Vibrios 1629 and 1641. Seven days growth on leptospira medium and guinea pig kidney about natural size.

FIG. 2. 3 day plain agar culture Vibrio 1700, 40th transfer, illustrating slightly convoluted forms. Dilute carbolfuchsin. $\times 1,500$.

FIG. 3. Young plain agar culture Vibrio 1629 showing flagella. Bailey's flagella stain. $\times 1,400$.

FIG. 4. Young plain agar culture Vibrio 1700. Bailey's flagella stain. $\times 1,400$.

FIG. 5. 3 day blood agar culture Vibrio 1629, sixth transfer, showing longer coiled forms and a portion of a clump in which the vibrios have fragmented. Giemsa stain. $\times 1,500$.

FIG. 6. 4 day blood agar culture Vibrio 1655, first transfer. Long and short coiled forms are illustrated. Giemsa stain. $\times 1,500$.

FIG. 7. 3 day plain agar culture Vibrio 1629, 35th transfer, showing extremely long forms as well as shorter types and illustrating early fragmentation. Giemsa stain. $\times 2,000$.

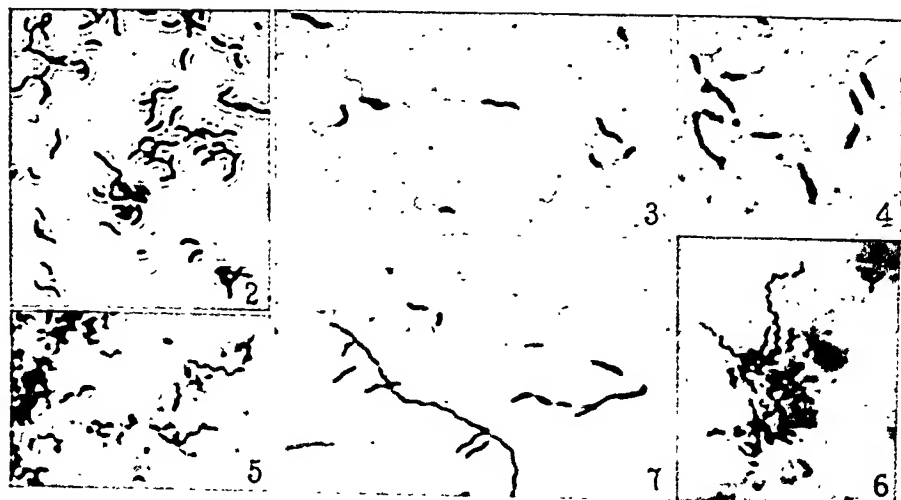


TABLE I
Bile Drained into Abdominal Cavity

Dog No.	Days after operation	Blood				Remarks
		Amount per 100 cc.			CO ₂ combin- ing power	
		Total non-protein nitrogen	Urea nitrogen	Chlo-rides		
1	0	27.7	10.5	480	48.3	Weight 11 kg., 600 gm.
	1	35.7	25.9	420	46.6	
	2	28.0	18.9	400	47.5	
	3	45.6	12.6	400	52.0	
	4	42.2	12.2	420	50.2	
	5	86.3	39.9	330	—	Died 5th day. No culture
2	0	40.0	21.7	520	41.9	Weight 14 kg.
	1	27.3	10.5	500	38.1	
	2	27.0	9.1	520	38.1	Appeared well Began feeding
	3	25.0	10.5	510	34.3	
	4	28.9	7.0	540	28.7	
	5	48.2	27.3	510	50.2	
	6	27.7	13.3	520	38.1	
	7	29.7	12.0	580	36.2	
	36	54.0	32.2	460	47.5	Reoperated upon 36 days later. Drained gall bladder into abdominal cavity
	37	126.0	80.5	400	20.9	Died within 48 hrs. Culture negative
3	0	31.4	13.3	450	34.3	Weight not recorded
	1	86.3	37.8	380	33.4	
	p.m.	137.0	92.4	350	—	Died within 36 hrs. Culture negative
4	0	21.8	7.4	450	38.1	Weight 18 kg., 400 gm.
	1	85.4	45.2	180	12.9	Died within 24 hrs. Culture positive
5	0	31.2	10.7	430	32.4	Weight not recorded
	1	30.0	13.5	350	40.9	
	2	98.0	73.9	600	38.1	Died within 48 hrs. No culture
	p.m.	110.0	85.4	410	—	
6	0	46.0	26.6	560	32.8	Weight 12 kg., 200 gm.
	1	57.0	24.3	630	27.3	
	p.m.	65.0	30.3	415	—	Died within 36 hrs. Culture negative

CHEMICAL CHANGES IN THE BLOOD OF THE DOG IN EXPERIMENTAL BILE PERITONITIS

BY ALLEN M. ZIEGLER, M.D., AND THOMAS G. ORR, M.D.

(From the University of Kansas School of Medicine, Kansas City, Kansas)

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General bile peritonitis in the human being is usually fatal if permitted to continue for a sufficient period of time without treatment. Horrall (1) gives the mortality rate as 50 per cent or more. The same author has found that bile from the gall bladder injected into the peritoneal cavity of dogs in quantity as large as 5 cc. per kilo of body weight will cause peritonitis and death within 24 hours. Wangenstein (2) found that bile peritonitis produced death in both dogs and rabbits within 24 hours.

In such a rapidly fatal condition it seemed probable that a study of the blood chemistry would show definite and constant changes. Definite changes have been noted in the chemistry of the blood of the dog in general peritonitis produced by ligating the appendix (3).

We report below changes noted in the non-protein nitrogen, urea nitrogen, chlorides and CO_2 combining power of dogs with bile peritonitis produced by draining bile from the gall bladder directly into the peritoneal cavity.

Methods

Dogs weighing 11 to 18 kilos were used. In every instance the operation was done under complete ether anesthesia with aseptic technic. The contents of the gall bladder were drained into the peritoneal cavity by cutting a slit in the fundus or by clipping off a small portion of the fundus. The common duct was not ligated. The animals were kept in metabolism cages and permitted to drink water as desired. Blood for chemical studies was drawn from the jugular vein before operation and after operation at least once each day until the death or recovery of the animal.

EXPERIMENTAL OBSERVATIONS

Drainage of bile directly from the gall bladder into the peritoneal cavity did not always produce death. Several of our dogs recovered

tures in four cases. In two cases no culture was taken. Anaerobic cultures were not made in this series.

DISCUSSION

After drainage of bile directly into the peritoneal cavity dogs died in from 1 to 5 days. It is evident that dog bile is quite toxic. Our animals lived somewhat longer than those of Wangenstein and Horrall, probably because the common duct was not ligated and the rate of flow of bile into the peritoneal cavity varied in different animals. We agree with these authors that sufficient bile, either injected or drained into the abdominal cavity of dogs, will invariably cause death. Apparently infection is not a contributing factor. We consider the positive cultures found in our animals a contamination.

The changes in the blood chemistry are similar to those found in experimental peritonitis in dogs. The increase in the non-protein and urea nitrogen in the blood is probably due to increased tissue destruction. The fall in chlorides is best explained by vomiting.

CONCLUSIONS

1. Changes in the chemistry of the blood of dogs with experimental bile peritonitis are here reported.
2. In all animals that died, a bile ascites was found.
3. Dogs dying of bile peritonitis showed a constant increase in the blood non-protein and urea nitrogen, and a fall in the chlorides.

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TABLE I—*Concluded*

Dog No.	Days after operation	Blood				Remarks
		Amount per 100 cc.			CO ₂ combin- ing power	
		Total non-protein nitrogen	Urea nitrogen	Chlo- rides		
7	0	27.5	12.6	510	36.1	Weight 10 kg., 200 gm.
	1	35.3	12.6	480	40.3	
	2	50.0	19.6	460	29.1	
	3	50.8	12.8	430	32.8	
	4	46.0	20.0	470	39.3	
	5	50.8	20.0	440	32.8	
	6	48.2	17.7	450	40.3	
	7	41.3	17.7	430	31.0	Dog killed. Abdomen full of bile-stained fluid. Began to eat on 7th day following operation and appeared improved. Culture negative. This animal probably would have recovered

completely without evidence of illness. Autopsy of these animals showed the gall bladder healed with no evidence of bile peritonitis. Dogs developing a bile peritonitis which caused death invariably showed definite changes in the blood chemistry. There was an increase in the non-protein nitrogen and urea nitrogen and a moderate decrease in the whole blood chlorides. The carbon dioxide combining power did not show any constant change. However, in three of the animals there was a marked decrease in the carbon dioxide combining power of the blood just before death.

Dog 2 recovered completely from the first drainage of the gall bladder into the peritoneal cavity. On the 36th day the abdomen was reopened and the gall bladder again drained. Death resulted within 48 hours. Dog 7 was apparently recovering when killed with chloroform on the 7th postoperative day. Definite changes were noted in the blood chemistry.

At autopsy all dogs showed the peritoneal cavity distended with bile-stained liquid. Culture from the ascitic fluid of dogs dead with bile peritonitis showed positive cultures in one case and negative cul-

result in protection against the infection in more than 50 per cent of rats. In those rats protected against the *Bartonella* anemia all elements of the spleen are regenerated, but in the rats which are unprotected the splenic pulp shows exhaustion atrophy. A study of the pathological changes in unoperated immature 3 week old rats infected with the *Bartonella* infection by injection of blood from an adult anemic rat indicated that the pulp cells of the spleen are severely injured in *Bartonella* anemia (6).

In the present studies the pathological anatomy of *Bartonella muris* anemia is described. An effort is made, where possible, to trace the pathogenesis of the disease.

Methods

A series of 15 rats were splenectomized and killed at intervals during the first 2 weeks after splenectomy. The intervals were determined by the degree of anemia so that the changes at different stages of the anemia could be studied. In addition, the pathological changes in a large series of rats dying of *Bartonella muris* anemia in the course of other experiments were studied. The pathology of *Bartonella* anemia in 3 week old rats with intact spleens that had died of anemia following injection of blood of adult splenectomized rats was also included in this study. Detailed protocols have been omitted. In all instances bone marrow was examined and routine hematoxylin-eosin stains used, except where otherwise indicated.

An anemia develops within 2 to 7 days following splenectomy in the adult rat. The red cell count and the hemoglobin drop at the same rate. Often within 2 or 3 days the count is less than one million and the hemoglobin less than 10 per cent. The blood shows poikilocytosis, anisocytosis, polychromasia and nucleated red cells. The *Bartonella* bodies appear as small diplococci and bacilli on the surface of the red cells and may number 15 to 20 in a cell. They have been described in considerable detail by Ford and Eliot (7), Mayer (8) and others.

At autopsy a rat dead from *Bartonella* anemia presents a striking picture.

The subcutaneous tissue and the fat have an icteric tint. The blood is watery and does not clot. The tissues and the organs of the body are strikingly anemic. Free fluid is present in varying amounts in the peritoneal cavity and effusions are found in the pleural and pericardial cavities.

STUDIES ON *BARTONELLA MURIS* ANEMIA

IV. PATHOLOGICAL CHANGES DURING THE ACUTE ANEMIA

By DAVID PERLA, M.D., AND J. MARMORSTON-GOTTESMAN, M.D.

(From the Laboratory Division, Montefiore Hospital, New York)

PLATES 32 TO 34

(Received for publication, March 26, 1931)

The occurrence of *Bartonella* anemia in the rat affords an excellent index of splenic function and opens a new avenue of approach in the investigation of certain phases of spleen physiology particularly as regards its rôle in infection and resistance. The rat possesses little hemolymph tissue (1) and, unlike other mammals, removal of the spleen results in almost complete removal of all splenoid tissue of the body. The pathology of *Bartonella muris* anemia has been briefly described by Lauda (2) and casual references to it have been made by other investigators (3, 4). Many phases of the pathological changes occurring in this disease have not been described. We studied the pathological anatomy of the *Bartonella* infection and the compensatory changes which occurred as a result of splenectomy. In previous studies we have demonstrated that the adult normal rat is a carrier of the *Bartonella muris* virus and that the peripheral blood is a source of infection (5). The blood of adult normal rats of carrier stock when injected into young immature rabbits produces in the rabbit a severe anemia with the appearance of *Bartonella* bodies in the red cells in large numbers. Diseases which severely injure the splenic pulp tissue may permit a flare-up of a latent infection of *Bartonella muris* anemia. *Trypanosoma lewisi* infection in the rat is accompanied at the height of the infection by the appearance of *Bartonella* bodies in the red cells and a moderate anemia. Oroya fever or *Bartonella bacilliformis* infection in human beings occurs generally among individuals whose spleens are severely injured from chronic malarial infection. We have found that small autotransplants of splenic tissue into the abdominal wall of rats inserted 7 weeks prior to splenectomy will

rather than a true lymphocytic response to the infection. Large quantities of blood pigment are seen in swollen endothelial and reticular cells.

Liver.—The changes in the liver can be followed more accurately. The organ is soft in consistency and yellowish in color. The lobular markings are not clearly distinguishable. On section the liver cells show varying quantities of fat droplets. There is a diffuse distribution of the lipoid material in the central and periportal areas. Scattered diffusely through the liver are areas of focal necrosis with no surrounding inflammatory reaction. The histogenesis of these lesions can be studied and different stages of the process may be observed in rats killed before the height of the anemia is reached. The Kupffer cells phagocytize red blood cells containing *Bartonella* bodies. They swell enormously so that a single Kupffer cell may fill an entire capillary lumen. Several such enormously distended cells are seen in Fig. 3. At a later stage the Kupffer cells proliferate and increase in number and small foci of Kupffer cells accumulate within the lumen of the liver capillaries compressing the surrounding liver cord. See Fig. 4. These engulf red cells and pigment. Gradually they undergo degeneration, disintegrate and focal necrosis develops. Nuclear debris can still be seen within these areas.

Occasionally small leucocytic foci are found in the liver of animals which are recovering from the anemia. The areas of necrosis heal, and repair occurs without scar formation.

The suprarenal glands are slightly enlarged and microscopically there is cloudy swelling of the cortical cells.

Kidneys.—The kidneys are the seat of severe degenerative changes. At autopsy they are pale and swollen, the markings are indistinct. Microscopically the glomeruli show large areas of necrosis with a marked diminution of the nuclear elements. In some glomeruli the endothelial cells are swollen, the capillaries are free of blood, occasionally a few red cells are seen in the space of Bowman, or a small accumulation of serum is present just beneath Bowman's capsule. The necrosis of the tuft commences in a few endothelial elements and the rapid disintegration of the cells may go on until an entire glomerulus is necrotic (Fig. 6). Rarely proliferation of the endothelium of the capsule of the tuft is seen. This is not indicative of inflammation but a part of the generalized endothelial hyperplasia. The lesions in the glomeruli are not prominent.

The tubules, however, show a very extensive lesion. Many of the tubules are completely necrotic. The epithelium lining the tubules shows no nuclear elements, the lumina are filled with debris and albuminous material (Fig. 7). The lesion is that of a severe nephrosis primarily with less prominent degenerative changes of the glomeruli. Occasionally a yellowish pigment is seen in the epithelium of the convoluted tubules. Fat droplets are present in the epithelium of the collecting tubules.

The severe nephrosis is due to the severity of systemic infection but the excretion of hemoglobin in large amounts must contribute to the lesion. Hemoglobinuria is a common symptom in severe instances of *Bartonella muris* anemia. At autopsy the bladder is filled with bloody urine. Cellular elements are not present, however.

Heart.—The myocardium is yellowish brown and fatty. Microscopic section reveals the presence of fat droplets within the heart muscle cells as is found in all severe anemias. The degree of myocardial fatty metamorphosis is directly proportional to the severity of the anemia. With the recovery of the animal no residual evidence of the degeneration of the muscle remains. No inflammatory lesions have been noted. The lesion represents a severe parenchymatous degeneration as a result of infection with marked fatty change secondary to an anoxemia resulting from the anemia.

Lungs.—The lungs show no changes other than pallor and a terminal edema. No inflammatory lesions of the upper respiratory passage are observed. However, in animals recovering from the anemia pneumonic lesions in the lungs are frequently seen. These pulmonary infections appear as small pulmonary abscesses with central necrosis. In other instances a mucoid exudate is present in the alveoli. It is composed of pale staining stringy material in which are scattered a few desquamated epithelial elements. In places the entire alveolus becomes filled with vacuolated epithelial cells. These changes are due to secondary infections independent of the *Bartonella* infection. In immature passively infected rats the pneumonic complications are more common and congestion of the vessels of the lung is prominent.

Spleen.—The changes in the spleen in *Bartonella muris* anemia may be studied in the 3 week old rat with intact spleen since at this age the rat may be infected with *Bartonella muris* anemia by injection of blood of an anemic adult splenectomized rat. In those rats that rapidly succumb to the anemia the spleen is swollen, soft and slightly enlarged. On section the pulp is quite pale. Microscopically the pulp contains large areas of necrosis. The follicles are distinguishable but not prominent. The necrotic zones are not surrounded by any cellular elements indicative of inflammatory reaction. The sinuses are distended, the endothelial elements are swollen and in some areas many endothelial cells have undergone disintegration. The focal areas of necrosis develop about such disintegrating elements. Phagocytosis of disintegrating red cells laden with *Bartonella* bodies by the endothelial elements is rapidly followed by necrosis of these cells. The rapidity of the process in the young rat, 24 to 48 hours, does not permit of a detailed study of the development of these lesions. In places small, dirty, bluish foci are seen within the necrotic areas. Fig. 1 illustrates the presence of large areas of necrosis in the spleen of a 3 week old rat dying of anemia produced by the injection of blood of an anemic rat. In young rats that survive the infection or live a few days, there is a definite reaction of the lymphoid tissue of the spleen. The spleen is larger than normal. Microscopically the follicles are prominent and show a marked increase in the pale staining reticular elements that appear as lighter zones about the lymphocytes. There is hyperplasia of the lymphoblastic elements in the germinal centers and a hyperplasia of the surrounding reticular cells. Secondary lymphoid follicles develop in extrafollicular areas. It is probable that the hyperplastic follicles are due to hyperactivity of reticular cells

the Kupffer cells show marked phagocytic activity and proliferate. Death of these elements follows as a focal necrosis. It is probable that capillary thrombi may contribute to the ischemia of these areas. In the lymph nodes and in the thymus the lesions are almost entirely a result of hyperactivity and exhaustion of endothelial elements and the thymus presents focal capillary thrombi and marked activity of the endothelial capillary elements such as are found in the Kupffer cells of the liver. The kidneys show two types of lesions, one dependent on endothelial destruction in some of the capillaries resulting in focal necrosis in the glomeruli; the other a severe nephrosis, a result of severe infection and the excretion of hemoglobin. So called idiopathic hemoglobinuria of human beings may not result in any demonstrable lesions at death but autopsies during attacks are rare. In rats the physiological injury to the capillaries even where no marked lesions are present is indicated by the appearance of hemoglobin in the urine and the excretion of the virus of *Bartonella muris*. Finally the hyperplasia of bone marrow is the expression of regeneration of the red cell elements.

The pathological changes in this infection are due to several influences. The parasite resides in the red cells of the host and produces a rapid destruction of these cells. The cellular debris that accumulates in the circulating blood must be removed and the enormous activity of the endothelial cells of the capillaries of many of the internal organs is indicative of the participation of these elements. The severe nephrosis may be referable to the liberation of toxic substances during the red cell destruction. The severe, rapidly developing anemia leads, by anoxemia of the tissues, to marked fatty degeneration of the parenchymatous tissue of the heart, liver and kidneys. The icterus is a manifestation of severe blood destruction, and of liver injury. In young animals the endothelial reaction in the spleen, liver, thymus and lymph nodes is analogous to the reaction of these elements in other parasitic blood diseases, as, for example, malaria, and in such metabolic diseases as Gaucher's disease. The severe endothelial lesions, the presence of focal necrosis in the liver and in some instances in the thymus does not indicate that *Bartonella muris* anemia is a disease of the reticulo-endothelial system as maintained by Cannon and others. The reaction of the phagocytic elements comprising this system must be looked upon as an attempt to get rid of foreign material.

It became apparent in studying the lesions of the kidney that the urine may be a source of dissemination of *Bartonella muris*. Eliot and Ford (9) have already shown that parasites will transfer it. Contamination of food by the urine of infected rats is conceivably another means of its transmission. To test the infectivity of the urine the following experiment was performed:

Three splenectomized rats were placed each in a specially constructed metabolism cage consisting of a large glass funnel at the narrow end of which was placed a glass plate so cut as to permit the escape of urine but not of feces. The end of the funnel fitted into a 50 cc. bottle. The urine was collected during the height of the anemia, neutralized and injected in amounts of 0.3 cc. into 3 week old rats. Two of three rats injected developed a severe anemia with the appearance of *Bartonella* bodies in the red cells.

From this experiment it is evident that the *Bartonella* bodies or the virus of the anemia is present in the urine and is capable of infecting a susceptible animal when injected into such animals.

Thymus.—The thymus gland is the seat of focal necrotic lesions. The lymphocytic elements are atrophic. The focal areas of necrosis are in the medulla. In places about the necrotic zones are thrombosed capillaries. (See Fig. 8.) The nature of these thromboses and their histogenesis can be determined by observing the early changes in some of the non-thrombotic capillaries. Occasionally marked swelling and proliferation of the endothelial elements of a capillary wall occur. Fig. 5 illustrates the reaction of the capillary endothelium. Several cells have desquamated into the lumen. The injured epithelial lining is probably the nidus of thrombus formation and the focal necroses are in part a result of impaired circulation.

Bone Marrow.—The bone marrow is deep red even in fatal cases, and an attempt at regeneration of the red cell elements is present. The bone marrow shows large numbers of erythropoietic elements. Many megaloblasts are present.

Lymph Nodes.—Many of the nodes show some degree of catarrhal lymphadenitis. That is, the endothelial elements of the sinuses proliferate and fill the sinuses. The follicles are not prominent. The endothelial elements form small sheets of cells.

DISCUSSION

This disease affects primarily the endothelial tissue of the body and to a lesser extent the reticular elements. In the spleen (of the young rat) the areas of necrosis arise in the endothelial elements lining the sinuses and perhaps in the reticular elements of the pulp; in the liver

EXPLANATION OF PLATES.

The tissues were stained with hematoxylin-eosin.

PLATE 32

FIG. 1. Spleen of a 3 week old unoperated albino rat, dead of *Bartonella muris* anemia. Large areas of necrosis in the pulp. Magnification 240 \times .

FIG. 2. Adult splenectomized rat, killed at the height of *Bartonella muris* anemia. Liver showing areas of focal necrosis. Note the absence of inflammatory reaction about the areas of focal necrosis. Magnification 240 \times .

PLATE 33

FIG. 3. Adult splenectomized rat, killed at the height of anemia. Liver—Kupffer cells enormously distended filling the lumen of the capillaries. The phagocytic activity of these cells is prominently illustrated. Magnification 1200 \times .

FIG. 4. Adult splenectomized rat, killed at the height of anemia. Liver—showing proliferation of Kupffer cells, dilating the capillary space, some undergoing necrotic change. Magnification 1200 \times .

FIG. 5. Adult splenectomized rat, killed at the height of anemia. Thymus—capillary wall showing swelling of endothelial cells, proliferation and desquamation. Magnification 1200 \times .

PLATE 34

FIG. 6. Adult splenectomized rat, killed at the height of anemia. Kidney—complete necrosis of glomerulus. Magnification 480 \times .

FIG. 7. Adult splenectomized rat, killed at the height of anemia. Kidney—nephrotic changes. The tubular epithelium shows marked degeneration, loss of nuclear elements, swelling and disintegration of the cytoplasm. Magnification 480 \times .

FIG. 8. Adult splenectomized rat, killed at the height of anemia. Thymus: medulla—showing focal necrosis. Note the thrombosis of the small vessel. Magnification 480 \times .

Bartonella muris anemia is primarily a disease of the red cells of the body, like malaria. The rôle of the spleen is a specific one in preventing the invasion of the red cells *en masse* by this virus. This protective action is not completely developed in the 3 week old rat since at this period the unsplenectomized animal is susceptible to infection. In the adult, however, infection cannot occur with the spleen intact. If it is removed then the infection influences, through the blood destruction that it causes, the endothelial elements of the body.

It is important to determine whether the function of the spleen in preventing *Bartonella* infection is taken over by any other group of cells or tissue and if so, the nature of the compensatory mechanism.

SUMMARY

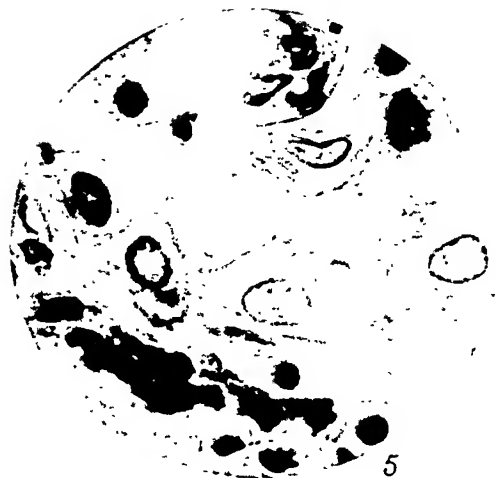
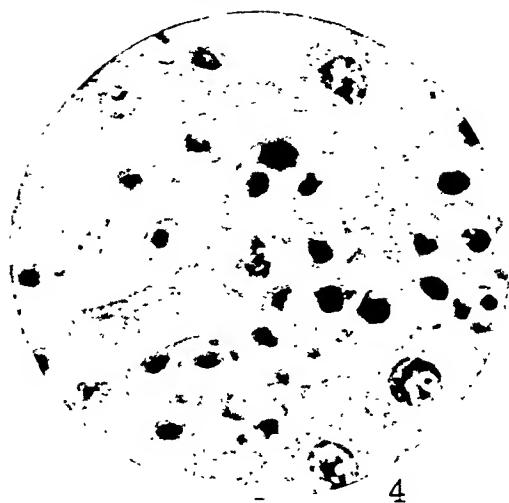
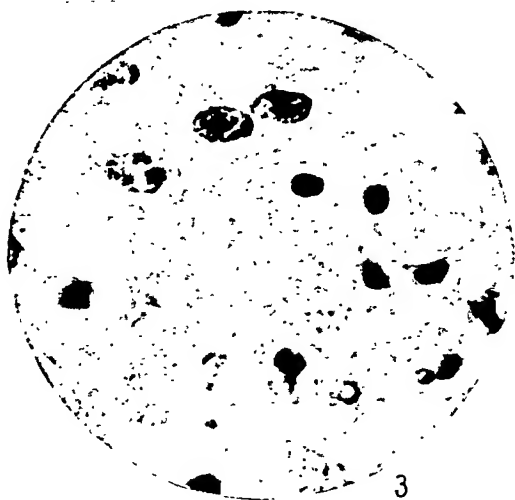
The pathological changes that follow a severe infection with *Bartonella muris* in the adult or young albino rat are: First, those that result from the release of large quantities of cellular debris in the circulating blood. There is phagocytic activity and hyperplasia of the endothelial elements of the liver, thymus, lymph nodes and, in the young rat, of these elements in the spleen, with resultant capillary thromboses and focal necroses. Second, changes result from the anemia as such—fatty metamorphosis of heart, liver and kidneys. Third, there is a severe nephrosis and in some instances a degenerative process in the glomeruli. In the bone marrow hyperplasia of erythropoietic elements occurs.

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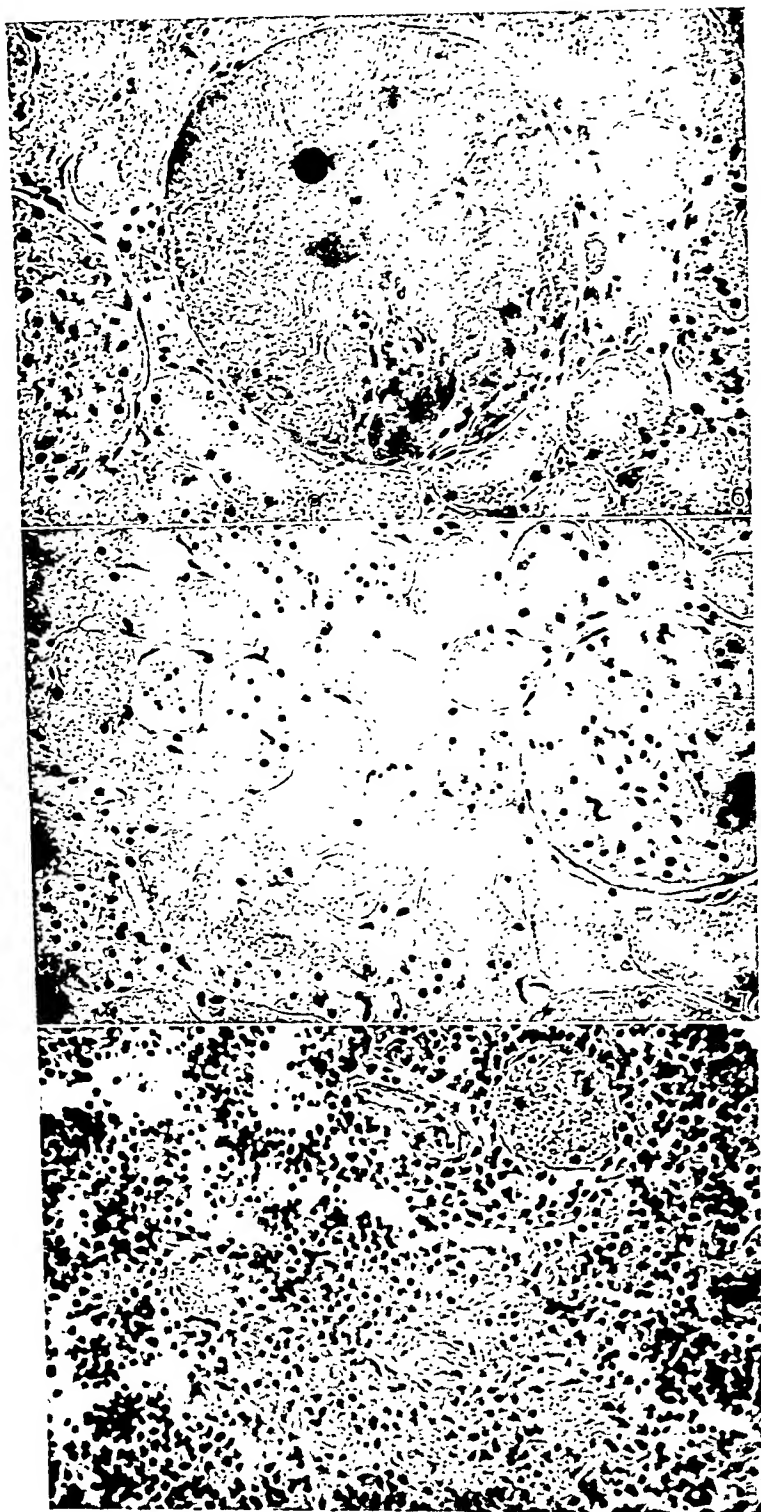
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(Perls and Marmonten-Gottesman: *Erythrocytes with anemia*. IV.)







killed in 9 months. 2 weeks before the rats were killed they were all injected with whole blood of an anemic rat. The blood counts and hemoglobin readings were recorded for 2 weeks after the injections. Careful autopsies were made on all the rats and routine sections of all the tissues were studied.

It was found that 6 weeks following splenectomy a rat may be reinfected with *Bartonella muris* anemia by injecting the blood from an anemic rat. However, all the rats tested 3 and 5 months after splenectomy remained resistant to the anemia and these rats could not be reinfected with the virus. These experiments suggested two explanations. First, that an active, severe infection resulted in a permanent immunity. Second, that some compensatory mechanism for the loss of the spleen had developed which protected the rat against the infection. Work by Ford and Eliot (3) on the nature of the immune reactions in this disease as well as our own observations would indicate that immune substances cannot be demonstrated in the peripheral blood following this disease. Ford and Eliot (4) have shown that the blood of a recovered rat or of an adult non-splenectomized rat contains no protective substances. Further, it has been observed that rats after recovery from one attack of anemia may be reinfected or may spontaneously relapse. None of the investigators who mention this fact state how long a time had passed after splenectomy. Our own observations indicate that when 6 weeks to 2 months has gone by the rat can no longer be reinfected and in all instances in the 3rd month, resistance is completely reestablished.

The Effect of a Severe Active Infection with Anemia in Young Unoperated Rats on the Subsequent Resistance to Reinfection Following Splenectomy

In an effort to determine if the resistance of rats to reinfection with *Bartonella* anemia 3 and 5 months after splenectomy is due to an active immunity resulting from a severe infection, the following experiment was undertaken:

Four 3 week old rats were injected with the blood of anemic adult splenectomized rats. All developed a severe anemia within a few days and recovered within a week. 2 months later they were splenectomized. All four developed a severe anemia with *Bartonella* bodies in the red cells.

STUDIES ON *BARTONELLA MURIS* ANEMIA

V. COMPENSATORY PHENOMENA FOLLOWING SPLENECTOMY IN THE ADULT ALBINO RAT

By J. MARMORSTON-GOTTESMAN, M.D., AND DAVID PERLA, M.D.

(From the Laboratory Division, Montefiore Hospital, New York)

PLATES 35 AND 36

(Received for publication, March 26, 1931)

In previous work the specificity of the spleen in the protective mechanism of the body to *Bartonella* infection was studied (1, 2). It was found that autotransplants of splenic tissue in the abdominal wall of rats afforded protection against the anemia in about 50 per cent of instances. In those rats that were unprotected against the anemia the regenerated transplants showed exhaustion atrophy of the pulp cells, but the lymph follicles remained intact. In those rats that were protected all the elements of the spleen regenerated. These experiments demonstrated that the function of the spleen can be assumed by a minute fragment of splenic tissue if it is completely regenerated and that innervation of the spleen plays no rôle in its specific protective action. In an attempt to determine the nature of the protective mechanism of the spleen, splenic extracts were made and implantations of fresh rat spleen were carried out. Aqueous and alcoholic extracts of cow's or pig's spleen or implantations of rat spleen afforded no protection against *Bartonella muris* anemia in splenectomized rats. It is possible that the rôle of the pulp elements of the spleen is dependent on its vital activity.

The experiments reported in the present communication were carried out to determine what compensatory phenomena develop in the rat as a result of splenectomy. Is the specific function of the spleen in its rôle in resistance assumed by any other organ or group of cells?

Method

24 adult albino rats were splenectomized. The course of the anemia was studied. 6 rats were killed in 2 months, 9 were killed in 5 months, and 9 were

The hemolymph nodes in the retroperitoneal region of the rat are hypertrophied in all the rats autopsied 3 and 5 months after splenectomy. On section the sinuses of the nodes are filled with blood, the endothelial elements are enlarged and a hyperplasia of these elements is present. The endothelial cells and many of the reticular elements in the depths of the nodes are distended with engulfed red blood cells and particulate hemosiderin.

The erythrophagocytosis is primarily limited to the endothelial elements but pigment is found in the reticular elements as well. Normally this degree of physiological activity is not observed in hemal node tissue nor in the spleen except in the presence of infection and marked red cell destruction.

Lymph Nodes.—In some instances a moderate degree of hyperplasia is present. In many instances, however, the endothelial elements of the sinuses have undergone hyperplasia and numbers of cells fill the lymph sinuses. In places the sinuses are replaced by sheets of sinus endothelium, which extend from the periphery toward the medullary portion of the node.

Thymus.—All the rats used in this experiment were 6 and 8 months old at the time of autopsy. The thymus is not enlarged. Microscopically the thymus is completely regenerated as in an immature rat. There is an increase in the small cells, and a more prominent regeneration of the reticulum. In some instances the lymph follicles are enlarged.

Liver.—The liver presents no gross changes, in most instances. On section, in some cases, fine gray specks are seen scattered throughout the liver. Microscopically there are accumulations of lymphocytes in the periportal area (see Figs. 1 and 2). These vary in size and in some cases are as prominent as lymphoid infiltrations of lymphatic leukemia of human beings. These foci are composed entirely of small lymphocytes and appear to arise in the reticular tissue about the periportal areas. The liver cells show no abnormalities. The Kupffer cells are small and normal in appearance.

Lungs.—The lungs show no gross changes. Microscopically there are no patches of pneumonia, the bronchi are normal. Scattered through the lung are foci of closely packed small round cells (lymphocytes) (see Figs. 3 and 4). These are grouped about bronchi and small

It is evident that an active induced infection in the 3 week old rat does not afford permanent immunity to the infection. At about 4 to 5 weeks of age, rats will spontaneously develop a mild degree of *Bartonella* infection with very little anemia (Jaffe and Willis (5)). Apparently the severity of the first infection does not influence the degree of resistance to future infection.

It is plain, therefore, that the resistance to reinfection with *Bartonella muris* which is present 3 months after splenectomy results from a compensatory mechanism which has developed during that time. The finding of Ford and Eliot (4) that a Wistar Institute non-carrier rat is susceptible to infection with *Bartonella muris* 9 months after splenectomy is not necessarily inconsistent with our findings. It is entirely probable that a compensatory mechanism to splenectomy develops only as a result of a physiological need and in the absence of the *Bartonella muris* virus such physiological compensation for the protective action of the spleen does not develop.

The Late Effects of Splenectomy. Morphological Changes 3 and 5 Months after Splenectomy in Bartonella Infected Albino Rats

All the rats in these experiments developed an anemia following splenectomy within the 1st week and recovered at the end of a month. After this period no spontaneous recurrences were noted. At the end of 3 and 5 months neither group could be infected with *Bartonella muris* by injecting large quantities of whole blood of anemic rats. The important morphological changes were found in (1) hemolymph nodes, (2) thymus, (3) liver, (4) lungs, (5) bone marrow and (6) lymph nodes.

Hemolymph Nodes.—The rat possesses one pair of hemolymph nodes behind the suprarenals which are very small and one pair in the posterior upper mediastinum, which are very difficult to locate (6). It has been shown by Warthin (7)* in 1901 that splenectomy in goats and sheep is followed rapidly by enlargement of lymph nodes and increase in the size and number of the hemolymph nodes. The rabbit, sheep, goat and guinea pig have much more hemolymph tissue than the rat. Warthin considered from his observations that the hemolymph tissue is accessory erythropoietic tissue.

* The literature on the compensatory growth of hemal nodes following splenectomy prior to 1901 is reviewed by Warthin.

their endothelial elements, the periportal accumulations of lymphoblastic tissue, the striking reticular and lymphoblastic hyperplasia of the peribronchial and perivascular regions of the lung in the absence of inflammation of the parenchyma, the regeneration and hyperplasia of all elements of the thymus must be considered as compensatory phenomena following the removal of the spleen. Whether the transient acute anemia resulting from the infection with *Bartonella muris* contributes to the lymphoblastic and reticular and endothelial hyperplasia found months later is doubtful though this can only be determined by studies on the late effects of splenectomy in carrier-free stock. That the phenomena following 3 to 5 months after splenectomy are compensatory for the loss of the spleen is strongly suggested by the fact that the rats can no longer be reinfected with the virus of *Bartonella muris* anemia, whereas reinfection may occur within the first 6 weeks following splenectomy. An active acute infection with the anemia of *Bartonella muris* leaves no permanent immunity as has been demonstrated in experiments on young rats with intact spleens, for if the spleen in such rats is removed 2 or 3 months later, the infection and anemia recur. The specific protective function of the spleen to the *Bartonella* anemia is taken over by other elements of the body in the absence of the spleen. From the observations of Ford previously cited, it is possible that this compensatory mechanism does not develop in the absence of a need for it. In *Bartonella muris*-free stock a rat may still be susceptible to infection months after splenectomy.

SUMMARY

In *Bartonella muris* carrier stock certain changes are observed in the lymphoblastic and reticular and endothelial elements after splenectomy and recovery from the anemia. These changes appear 3 to 5 months after splenectomy and are associated with immunity to further infection with *Bartonella muris*. The changes consist primarily of hyperplasia of hemolymph tissue, hyperplasia of reticular and endothelial elements of lymph nodes, the formation of lymphoblastic foci periportally in the liver and peribronchially and perivascularly in the lung, regeneration of all elements of the thymus and marked hyperplasia of all elements of the bone marrow (increased hematopoiesis).

pulmonary vessels. The surrounding tissue shows no evidence of inflammation. These peribronchial and perivascular lymphocytic elements are not an expression of inflammation but of lymphoblastic hyperplasia and metaplasia of peribronchial reticular tissue. Reticular elements are numerous in the lymphocytic accumulations. In places they form clumps of 4 or 5 cells with fused cytoplasm. Some of the reticular elements contain blood pigment.

Bone Marrow.—The bone marrow of the long bones is a deep red. Very little fat is present. On section there is a striking degree of hyperplasia of all elements. Erythropoiesis and myelopoiesis are markedly stimulated. Figs. 5 and 6 show illustrative areas from normal and hyperplastic bone marrow of the rat. In many sections of the marrow large numbers of megakaryocytes are present (see Fig. 6).

DISCUSSION

These studies indicate that within 3 to 5 months following splenectomy in albino rats of *Bartonella muris* carrier stock, the following changes have occurred: (1) Moderate hyperplasia of lymph tissue of lymph nodes; (2) hyperplasia of hemal nodes, with marked hyperplasia of the endothelial elements of these nodes, and erythrophagocytosis by these cells; phagocytosis of blood pigment by the reticular elements of these nodes; (3) hyperplasia in the thymus of small cells and reticular cells, less in degree than that found in rats following suprarenalectomy; (4) marked hyperplasia of endothelial elements of the lymph nodes; (5) periportal accumulations of lymphocytes in the liver apparently arising from the reticular tissue at these sites; (6) peribronchial and perivascular accumulation of lymphocytes in the lung in the absence of all inflammatory changes in the parenchyma of bronchi; increase of the reticulum of the peribronchial areas; and (7) hyperplasia of all hematopoietic elements in the bone marrow with an enormous increase in the bone marrow giant cells, the so called megakaryocytes.

The hyperplasia of the bone marrow may be indicative of continued compensatory mechanism secondary to the anemia following the *Bartonella* infection. These rats, however, had been free of the active infection for a period of 2 to 4 months prior to autopsy. The hyperplasia of the hemolymph nodes with the intense physiologic activity of

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EXPLANATION OF PLATES

PLATE 35

FIG. 1. Adult splenectomized rat. Killed 5 months after splenectomy. Liver—showing periportal accumulations of lymphocytes. Magnification 100 \times .

FIG. 2. Same as Fig. 1. Higher magnification. 240 \times .

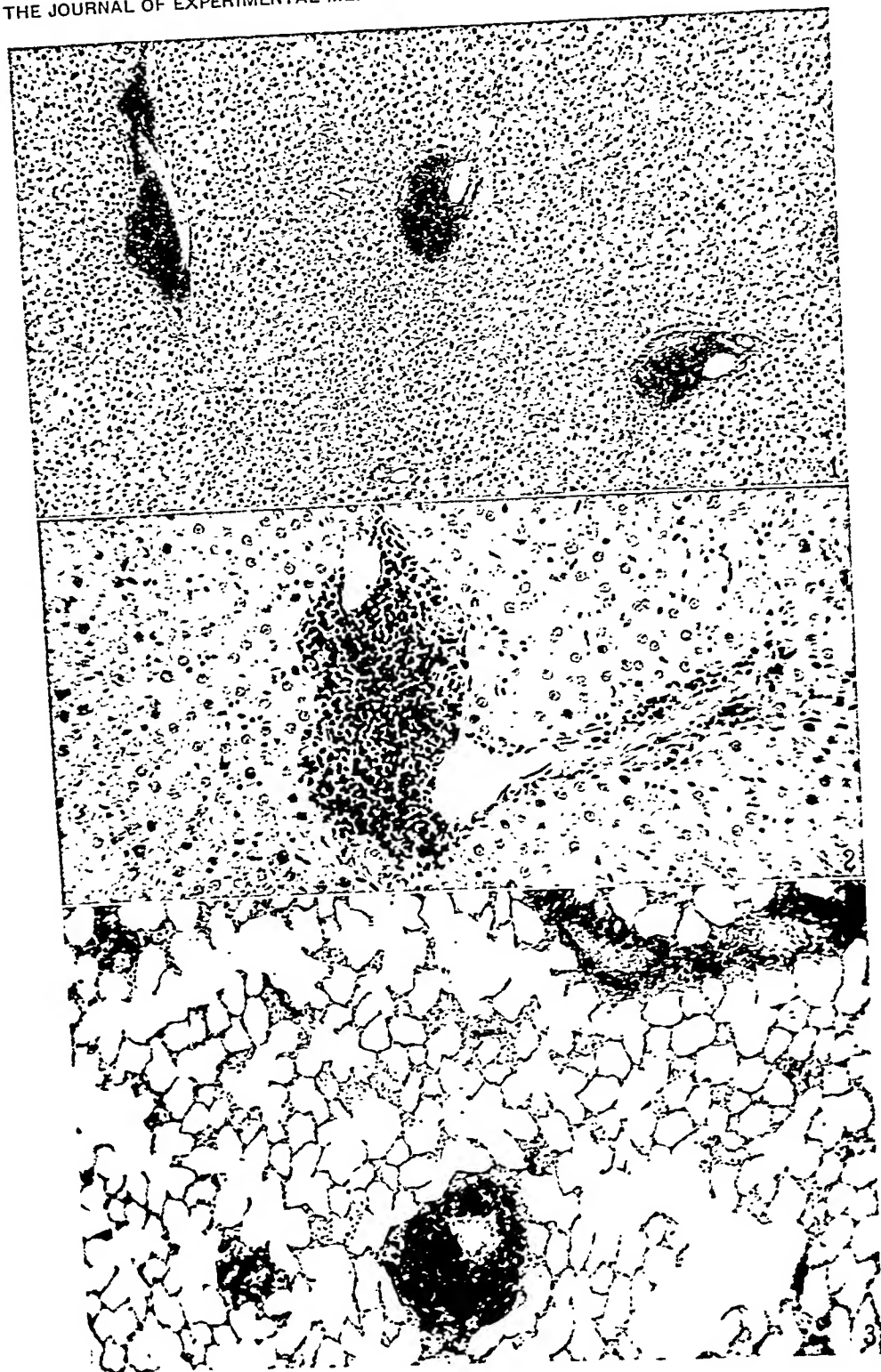
FIG. 3. Adult splenectomized rat. Killed 5 months after splenectomy. Lung—showing peribronchial and perivascular lymphocytic accumulations. Note the absence of any pulmonary infection. Magnification 100 \times .

PLATE 36

FIG. 4. Same as Fig. 3. Higher magnification of one perivascular lymphocytic nodule. Note the uniformity of cells. Magnification 240 \times .

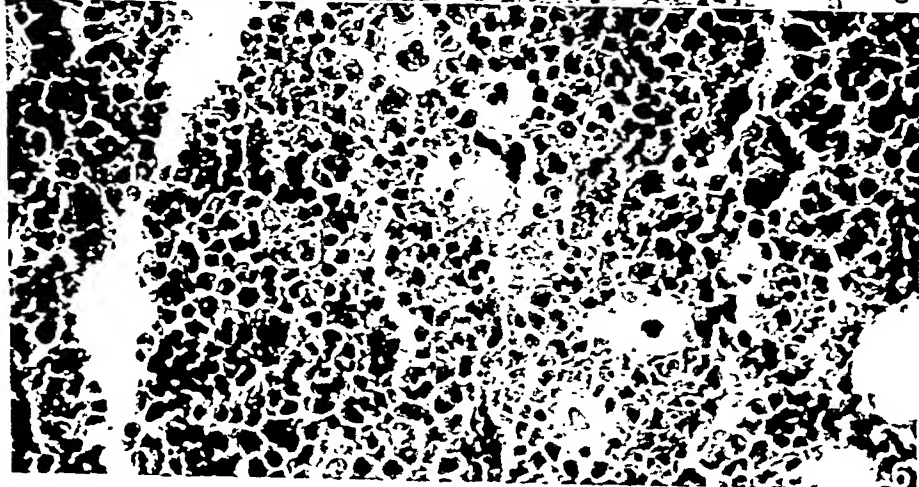
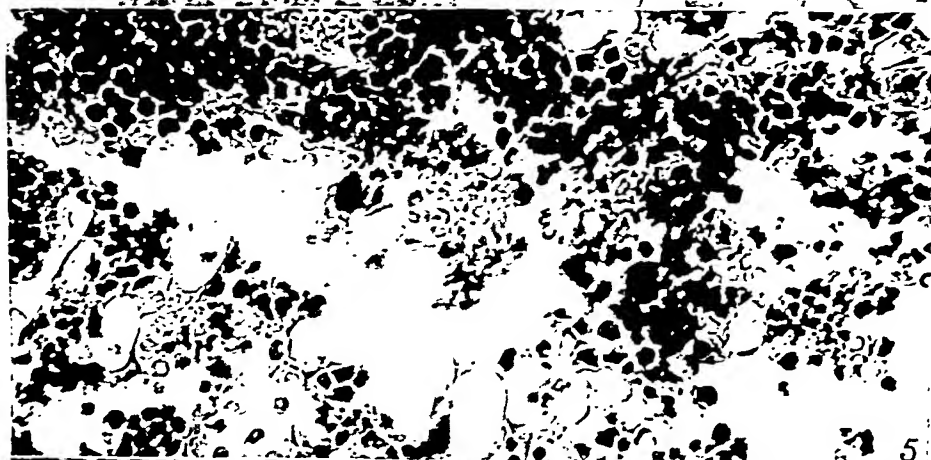
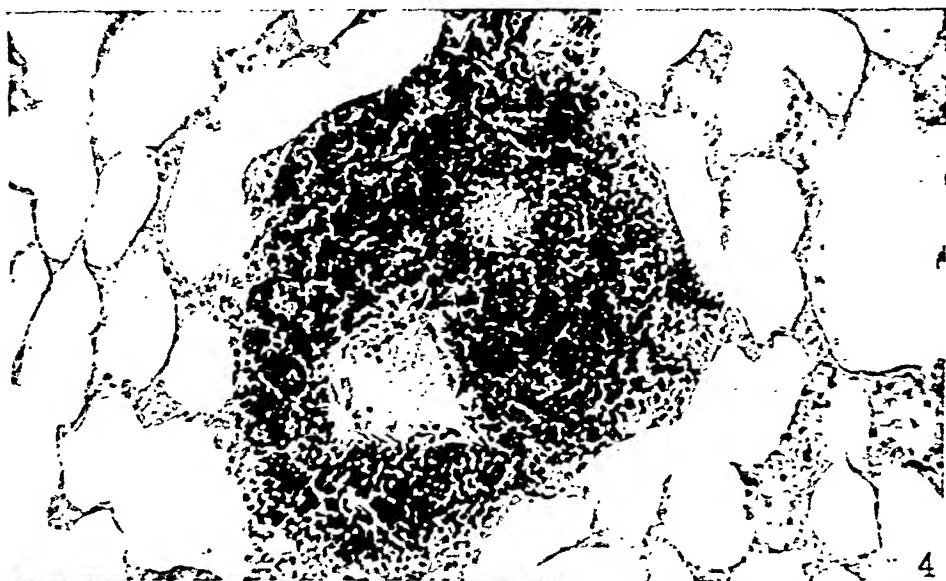
FIG. 5. Adult splenectomized rat. Killed 5 months after splenectomy. Bone marrow—showing marked hematopoiesis. Note the large number of megakaryocytes. Magnification 440 \times .

FIG. 6. Normal rat, 8 months old. Bone marrow—compare with Fig. 5. Magnification 440 \times .



(Marmorestein-Gottsmann and Perl: *Felinele wach anemie*. V)





(Marmarston-Gottman and Perl: *Paraneoplastic myeloid anemia*. V)

ACTIVE IMMUNIZATION AGAINST POLIOMYELITIS

The purposes of the following experiments were: (1) the production of active immunity against poliomyelitis by the least possible number of injections; (2) the determination of the minimal quantity of serum required to protect an animal against the dose of virus given; and (3) the determination of the optimum method and time of administration of the serum.

Technique

At each inoculation the material was injected in one piqûre. Except where otherwise stated, the virus was injected into the skin, although some of it infiltrated more deeply. Serum was administered subcutaneously in one piqûre.

Throughout these experiments, active poliomyelitis virus was used both for skin and intracerebral inoculation. Glycerinated cord was used, which was obtained from animals prostrate in 6 to 8 days after inoculation with "Fl. mixed virus." This virus was obtained from The Rockefeller Institute, where it was developed from the passage of pooled specimens of M.A. and K. virus (14). At intervals the potency was checked in this laboratory, and it was found that 0.01 cc. of a 5 percent glycerinated suspension produced prostration in from 10 to 12 days. Pooled human convalescent serum, which had been collected some 9 months previously, and kept at 4°C., was used. Its neutralizing power had been established in monkeys. Throughout the course of vaccination, the animals were observed for mild symptoms of the disease.

Inasmuch as Romer and Joseph (10) claimed that it took 26 days for immunity to develop, tests were not carried out until more than a month after the last injection. Control animals received a quantity of serum equal to the largest volume given to any of the experimental animals. In this way the retention of any passive immunity was controlled.

In testing the immunity, the so called "*in vitro*" test was used, by which is meant the ability of a serum from the test animals to neutralize a given quantity of virus. Stewart and Rhoads (7) found this procedure to be more delicate than the direct intracerebral inoculation of the experimental monkey with the virus. It was carried out in the usual way. Sufficient serum, taken from the treated animals, was added to the virus to make 1 cc., and after mixing well, incubation was carried out at 37°C., for 2 hours. The mixture was then kept overnight at 4°C., and injected intracerebrally into another animal.

EXPERIMENTAL

Experiment I.—A series of five monkeys received the equivalent of 20 to 30 cc. of 5 per cent active poliomyelitis virus emulsion, in one or two injections. Actually 10 per cent, or 20 per cent suspension was given to facilitate the injection of such large quantities of virus. In Table I the equivalents of 5 per cent emulsion have been calculated in each case to make the figures comparable with others in

ACTIVE IMMUNIZATION AGAINST POLIOMYELITIS IN MONKEYS*

By MAURICE BRODIE, M.D., AND ALTON GOLDBLOOM, M.D.

(From the Department of Experimental Medicine, McGill University, Montreal, Canada)

(Received for publication, March 11, 1931)

Although the active immunization of monkeys against poliomyelitis has been attempted many times with attenuated and chemically inactivated poliomyelitis virus (Landsteiner and Levaditi (1), Kraus (2), Zappert *et al.* (3), Abramson and Gerber (4)), success has been achieved only with living virus (Flexner and Lewis (5), Aycock and Kagan (6), Stewart and Rhoads (7), Rhoads (8)). However, the danger of infection occurring during the course of treatment is ever present, (Thomson (9), Aycock and Kagan (6)). Therefore, the purpose of this work was to attempt active immunization with active virus, obtained from monkeys prostrate with poliomyelitis, in from 6 to 8 days, together with sufficient human convalescent serum to add to the safety of the method.

Flexner and Lewis (5) were the first to confer active immunity against poliomyelitis to monkeys. They used subcutaneous injections of active poliomyelitis virus emulsion. Later Aycock and Kagan (6) used the intradermal route with success, while Stewart and Rhoads (7), in their experiments, found the intracutaneous injection superior to the subcutaneous for immunization.

A combination of immune serum and virus was used by Romer and Joseph (10, 11) and by Thompson (12), but the only serious attempt to produce active immunization with such material was made by Rhoads (13). He used equal parts of 5 per cent virus emulsion and immune serum, that had been in contact an hour. Several series of animals were treated subcutaneously and intradermally, either with two large injections, or multiple small inoculations.

* This research was made possible through the generosity of Mr. Russell Cowans and Mr. Alex. Christmas.

the literature. The virus was administered intradermally, while the serum was given subcutaneously, either with, or subsequent to, the injection of the infective material.

Results.—A study of Table I will show the antibody production of these animals against 0.05 cc. of 5 per cent active poliomyelitis cord emulsion (Neutralization Test 1). The sera of four out of five animals neutralized the virus. When the virus and the serum mixture of each of these was injected into another monkey, no symptoms occurred. The animal which received the serum from Animal 5-7 developed weakness of the right leg, which cleared up within 2 weeks. Three of the animals, Nos. 5-0, 5-4, and 5-8, whose serum was tested against 0.1 cc. of the same virus emulsions, failed to neutralize that amount (Neutralization Test 2). Monkey 5-7 was rendered prostrate on the 9th day after a direct intracerebral inoculation of 0.05 cc. of the same virus.

Four out of five animals failed to respond to 0.05 cc. of a 5 per cent suspension of active virus, which rendered the control prostrate in 8 days. It can be presumed that the fifth animal partially resisted the virus, for when the virus and serum mixture was injected into a monkey, only transient symptoms occurred after an incubation period of 18 days.

Monkey 1-18 was not tested against 0.1 cc. of 5 per cent virus. The remaining three were unable to resist that amount of cord suspension, as shown by Neutralization Test 2 in Table I.

The experience with Monkey 5-7 illustrates what has already been cited in the literature (Aycock and Kagan (6) and Rhoads (8)), namely, that the neutralization test is more sensitive for the demonstration of immune bodies than direct intracerebral inoculation.

According to the above experiments, virus together with human convalescent serum can produce immunity. It was important, therefore, to ascertain the minimal quantity of serum necessary to protect an animal against the disease during vaccination, without interfering with the immunizing power of the virus. Therefore, with a fixed amount of virus, varying quantities of immune serum were used.

Experiment II.—1 gm. of spinal cord, equivalent to 20 cc. of a 5 per cent suspension, was emulsified in 8 cc. of distilled water, and administered to each of six monkeys intradermally (Table II). The first received only the virus intradermally. The second and third received the virus with 2.5 cc. and 5 cc. of serum respectively, subcutaneously. The fourth animal had the virus and 6 cc. of serum. The last two were not injected with the virus and serum at the same time. One

TABLE I

Immunization					Neutralization Test 1			Neutralization Test 2			
Monkey No.	No. of injections	Total virus 5%	Serum	Course of immunization	Symptoms during immunization	Amount of virus 5%	Serum	Result	Amount of virus 5%	Serum	Result
5-0	3	28	10 cc.	2 cc. of 20% virus intradermally. In 6 days 10 cc. of 10% virus intradermally. In 1 day 10 cc. serum subcutaneously	None	0.05	0.95	Immune	0.1	0.9	8 days, weakness arms. 9 days—prostrate
5-1	2	24	7	6 cc. of 20% virus intradermally. In 2 days 7 cc. serum subcutaneously	None	0.05	0.95	Immune*	0.1	0.9	9 days—weak. 10 days—prostrate
5-7	2	26	9	4 cc. of 20% virus intradermally, plus 6 cc. of serum subcutaneously. In 6 days 5 cc. of 10% virus intradermally, plus 3 cc. serum subcutaneously	None	0.05	0.95	Symptoms at 18 days, with weakness of right leg. All cleared up	—	—	—
5-8	1	20	6	5 cc. of 20% virus intradermally, plus 6 cc. serum	None	0.05	0.95	Immune	0.1	0.9	10 days, both arms paralyzed. 11 days, prostrate
5-1-18	2	24	5	12 cc. of 10% virus intradermally. In 4 days 5 cc. serum subcutaneously	None	0.05	0.95	Immune	—	—	—
2-1	—	—	10	Control	—	0.05	0.95	Prostrate—8 days	0.02	0.98	Prostrate 9 days

* Died of intercurrent infection at end of 6 weeks.

§ Tested by *in vivo* tests with 0.05 cc. virus intracerebrally, developed paralysis 9th day. Necropsied.

‡ No. 1-18 died of intercurrent infection at end of 9 weeks.

The next experiment was to test the safety of the same proportions when the virus was given first, followed some days later by the serum (as indicated by the experience with Monkey 6 in our Table II), or when the serum was given first, followed later by the virus.

Experiment III.—Two monkeys (Table III) were used for this experiment. The first received 1 gm. of virus, followed in 3 days by 6 cc. of serum, while the other was given 6 cc. of serum, and 3 days later, a gram of virus.

Results.—Neither animal developed symptoms. Therefore, since we used a highly active virus and whereas, as in the case of Monkey 1-36, the virus was allowed to act 3 days before the serum, the administration of 6 cc. of serum with a gram of virus may be considered innocuous under the conditions outlined.

The next step was to determine the effects produced when the above method of administering these materials was reversed. Therefore,

TABLE III

Monkey No.	Virus	Serum	Method	Result
# 1-36	gm. 1	cc. 6	1 gm. virus given intradermally. In 3 days 6 cc. serum subcutaneously	No paralysis
1-34	1	6	6 cc. serum subcutaneously. In 3 days 1 gm. virus intradermally	No paralysis

* Died intercurrent infection at the end of 4 weeks. Histological sections ruled out poliomyelitis, as did a monkey transmission of cord.

the infective substance was injected subcutaneously, and the serum was given intradermally, presuming thereby that the virus was absorbed more rapidly than the serum.

Experiment IV.—One monkey was given a gram of virus subcutaneously, and 6 cc. of serum intradermally.

Result.—In 5 days the animal was paralyzed. Therefore, the quantities of virus and serum that had proved innocuous, using the former intradermally, and the latter subcutaneously, were infective when the virus was given subcutaneously, and its serum intradermally.

Having determined that 6 cc. of serum given subcutaneously rendered the intradermal inoculation of 1 gm. of virus innocuous, the next step was to test the immunizing power of virus and serum in these amounts.

received 4 cc. of serum 4 days earlier than the virus, while the other had 6 cc. of serum 3 days subsequent to the virus inoculation.

Results.—The first two animals succumbed to poliomyelitis within 9 days, while the third, which had been given 5 cc. of serum, fell ill on the 12th day, and was prostrate the 19th day. The fourth animal, which had received 6 cc. of serum, resisted the disease. The fifth animal, which had received 4 cc. of serum before the virus, succumbed to the disease on the 6th day, while the sixth monkey, which had been given 5 cc. of serum 3 days after the virus, remained well.

The above experiment indicates that, by this method of administration, 6 cc. of immune serum is required to protect a monkey against a

TABLE II

Monkey No.	Virus amount	Serum amount	Combination used	Result
	gm.	cc.		days
1	1	—	Virus intradermally	8—paralysis 1 arm. 9—died
2	1	2.5	Virus intradermally plus serum subcutaneously	8—paralysis right arm. 9—died
3	1	5	Virus intradermally plus serum subcutaneously	12—weakness right arm. 19—prostrate
4	1	6	Virus intradermally plus serum subcutaneously	No paralysis
5	1	4	Serum subcutaneously. In 4 days—virus intradermally	4—weakness left arm. 5—prostrate. 6—died
* 6	1	5	Virus intradermally. In 3 days—serum subcutaneously	No paralysis

* Died at end of 1 month of tuberculosis—no lesions of poliomyelitis.

gram of virus given intradermally. This is in contradiction to the results of Rhoads (8), who injected 16 cc. of virus emulsion (0.8 gm. of cord) intradermally into each of a series of four monkeys. None of his animals developed the disease. This discrepancy may be accounted for by the manner of injection of the virus, as many piqûres by Rhoads, and as one by us. Thus, in this work, more virus infiltrated the subcutaneous tissues (on account of the size of the dose given), thereby allowing more rapid absorption and more likelihood of infection.

The proportions of virus and serum that proved innocuous when administered simultaneously, were 6 cc. of serum to each gram of virus.

or serum 3 days after virus) induced sufficient immunity to resist two and one-half times the dose of virus that paralyzed the control animals. With specimens of this virus, infection had been produced with doses as small as 0.01 cc. of a 5 per cent suspension.

Immunity is a relative thing, and none is probably so great that it cannot be broken down by a large amount of virus. Indeed, Aycock and Kagan (6) have reproduced poliomyelitis in animals by using, at a second injection, large amounts of virus. Therefore, an immunity to several lethal doses may be considered definite, and perhaps useful.

In the two series, immunization was carried out on eight animals. Six of these resisted 0.05 cc. of 5 per cent virus emulsion, and the other two partially resisted as indicated by the prolonged incubation period and the milder attack in the test animals, as compared with the controls. Using larger amounts of virus, Rhoads (13) did not obtain as complete immunity, for only half of his monkeys resisted 0.01 cc. of a 5 per cent filtrate. However, he used more serum in proportion to virus, and in addition combined them. In this way he had complete neutralization as checked by intracerebral test.

Todd (15), Andrewes (16), and Long and Olitsky (17), using vaccine virus, and Schultz *et al.* (18) and Olitsky *et al.* (19) poliomyelitis virus, have shown that neutralization of the virus with immune serum does not destroy the virus. Yet, the fact that virus can be recovered from a combination with its serum, is no indication that such a mixture always dissociates sufficiently in the body to immunize efficiently. Only when the serum is not in excess is this possible. This has been pointed out by Zinsser and Tang (20), who produced active immunization against herpes virus with virus emulsion and immune serum. They concluded as follows: "Active immunity can be attained only when some degree of reaction to the living virus has occurred. Rabbits which survived neutralized serum-virus mixtures did not acquire immunity." Similarly Rhoads (21) used vaccine virus with immune serum, in rabbits, in such quantities as were innocuous intradermally. He found that an excessive amount of immune serum rendered the mixtures ineffective. Therefore, by using less serum than Rhoads, and yet sufficient to render the procedure safe, a greater degree of immunization has been obtained by us.

Experiment V.—Three animals, each of which was injected intradermally with a gram of virus made up to 8 cc. of emulsion, received 6 cc. of serum subcutaneously. The first received the serum 3 days before the virus inoculation, the second at the same time, while the third received serum 3 days after the infective material. Again, the neutralization test was used, the serum of the treated animals being tested against 0.05 cc. of virus emulsion. Not until 6 weeks after the last injection was the test made, thereby guarding against any residual passive immunity from the serum.

TABLE IV
Neutralization Test

Monkey No.	Process of immunization	Virus 5%	Serum	Result
		cc.	cc.	
1-34	6 cc. serum subcutaneously. In 3 days 1 gm. virus intracutaneously	0.05	0.95	Partial protection. Incubation 12 days, paralysis hind legs, and later partial of forearms. Survived. Recovering
1-35	1 gm. virus intradermally. 6 cc. serum subcutaneously	0.05	0.95	Immune
# 1-36	1 gm. virus intradermally. In 3 days 6 cc. serum subcutaneously	0.05	0.95	Immune
1-42	Control	0.02	0	9 days weakness right arm. 12 days—prostrate
1-43	Control	0.02	0	8 days weakness right arm. 10 days—prostrate
1-45	Control	0.02	0	12 days prostrate

Neutralization tests carried out 5 weeks after completion of course of vaccination.

Died at the end of 4 weeks of intercurrent infection.

Results.—(Table IV.) The serum of Animal 1-34, which received serum first and the virus later, gave doubtful protection. The test animal developed complete paralysis of the hind limbs, and partial of the upper extremities, but survived, and is recovering. The other two monkeys resisted the virus. The controls succumbed in from 10 to 12 days to 0.02 cc. of this virus emulsion.

In this small series, serum given subcutaneously with or after the injection of virus, was more effective than when the serum was given first. Moreover, a gram of virus with 6 cc. of serum administered in either of the more effective ways (*i.e.*, virus and serum simultaneously,

CONCLUSIONS

1. A combination of poliomyelitis virus and specific human serum is effective for the production of active immunity.
2. For each gram of active virus given intradermally as an emulsion, 6 cc. of human immune serum, injected subcutaneously, was required in our experiments to protect a monkey from paralysis. Some degree of active immunity was induced.
3. Immunity, without symptoms of the disease, was secured when the serum was given at the time of inoculation, or within 3 days preceding or following inoculation of the virus.
4. For the production of immunity, virus, preceded by serum administration, is probably less effective than when it is given simultaneously with, or before, the injection of serum.
5. The virus neutralization test is more sensitive than the direct intracerebral test for determining the production of immunity.

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host from the standpoint of the character of the blood picture before inoculation. These two aspects of our work will be reported later.

The present paper contains the results of successive observations on the peripheral blood picture of rabbits inoculated with a transplantable malignant neoplasm. The tumor has been carried in this laboratory for 10 years and has been used in many experiments under diverse circumstances (10); it is considered to be of epithelial origin.

Material and Methods

The material used in this study was derived from observations on 9 groups of rabbits, a total of 78 animals. The dates of inoculation and other data are given in Table I.

TABLE I

Group	No. of rabbits	Tumor inoculation	First blood examination	Last blood examination	Number of blood examinations*	
					Before inoculation	After inoculation
I	10†	Nov. 17, 1927	Oct. 24, 1927	Jan. 18, 1928	4	9
II	5	Jan. 5, 1928	Dec. 12, 1927	Feb. 14, 1928	9	19
III	9	Feb. 24, 1928	Feb. 8, 1928	Apr. 25, 1928	4	9
IV	10†	Apr. 20, 1928	Apr. 6, 1928	June 20, 1928	4	9
V	10	Nov. 22, 1928	Nov. 2, 1928	Jan. 21, 1929	4	8
VI	5†	Jan. 4, 1929	Dec. 29, 1928	Mar. 8, 1929	3	8
VII	12	Nov. 19, 1929	Oct. 29, 1929	Jan. 13, 1930	6	15
VIII	12	Jan. 14, 1930	Dec. 31, 1929	Mar. 18, 1930	5	9
IX	8	Jan. 14, 1930	Dec. 31, 1929	Mar. 18, 1930	5	9
Total. . . .	81				44	95

* In certain series, additional counts prior to the selected preinoculation period as well as the extra counts on a few animals kept 10 weeks after inoculation are not included.

† One rabbit each of Groups I and IV omitted because of a complicating nephritis; one animal of Group VI omitted because it was killed for transfer material before the end of the experiment.

The rabbits were adult male animals approximately 6 to 8 months of age at the time of inoculation. They were representative of the usual stock received from dealers and may be described as brown, black, and Flemish crosses. In the case of Group IX, the rabbits were born and raised in this laboratory; their ages varied from 6 to 12 months and for the most part, they also were of hybrid stock. Dur-

STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

VII. OBSERVATIONS ON RABBITS INOCULATED WITH A TRANSPLANTABLE MALIGNANT NEOPLASM

By LOUISE PEARCE, M.D., AND ALBERT E. CASEY, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, March 13, 1931)

Previous papers of this series contain successive observations on the blood count of groups of normal rabbits examined over prolonged periods of time (1, 2, 3, 4, 5). The striking feature brought out by the analysis of these results was the character of the numerical variations shown by the several classes of cells and by the hemoglobin content; it appeared that certain of these variations were of an orderly nature rather than chance occurrences and that in many instances, they appeared to be related to environmental (seasonal) conditions. A further statistical analysis showed, moreover, that a significant association existed between the variations of certain cells while in other cases, no relation whatever was demonstrated (6). The magnitude of the variations, it should be pointed out, was within the limits of what is usually considered normal.

The object of the observations on normal rabbits was not only to obtain information on the character of the blood picture with respect to environmental conditions, but in addition, to provide a background for similar observations in experiments dealing with the reaction of rabbits to various disease agents. The agents employed in these experiments were *Treponema pallidum* (7), virus III disease of rabbits (8), and a transplantable malignant neoplasm (9). In connection with these studies on the blood picture of inoculated rabbits, parallel observations have also been made on tissues involved in the disease process, the idea being that the findings in the one might be related to those of the other and thus open up a method of experimental approach to the question of the nature of the host's reaction. Still other studies have been carried out with respect to the reaction of the

the percentage deviation from so called standard values (1, 2). These percentage values were then combined in the form of an algebraic sum for each of the four animal groups and mean group values for each week were then calculated. By this method, individual animal and series peculiarities are minimized and in addition, the final figures may be considered as resembling smoothed values.

RESULTS

The results of nine experiments dealing with the peripheral blood cytology in rabbits inoculated with a transplantable malignant neoplasm are presented in a series of curves contained in Text-figs. 1 to 8. Because of space limitations, it has not been possible to include either the figures of individual observations or the combined weekly values. The curves represent in the form of mean percentage deviations from so called standard values, successive week to week levels of the eight blood elements studied, that is, the red blood cells, the hemoglobin content, the total white cells, the neutrophiles (pseudo-eosinophiles), the basophiles, the eosinophiles, the lymphocytes, and the monocytes respectively.¹ Each chart comprises four curves corresponding to the four groups in which the rabbits have been classified, that is, those animals which died as a result of the malignant disease, those which probably would have died had the experiments been continued, those which probably would have recovered, and finally, the recoveries or those rabbits in which no tumor was found at conclusion of the experiments.

As determined by clinical examination, a definite primary tumor developed in each rabbit with six exceptions, that is, one animal each

¹ The standard values used in this analysis are derived from 1110 blood counts on 174 normal rabbits (1, 2) and are as follows:

	<i>per c.mm.</i>
Red blood cells.....	5,200,000
Hemoglobin.....	63%
White blood cells.....	9560
Neutrophiles.....	4340
Basophiles.....	950
Eosinophiles.....	215
Lymphocytes.....	3050
Monocytes.....	1000

The curves of the present paper are drawn on the same scale as those which represent the findings in groups of normal rabbits observed for long periods of time and which were analyzed on the basis of the above values (2, 3, 4, 5).

ing the entire observation period, each animal was caged separately; the diet consisted of hay, oats, and cabbage.

The tumor inoculations were carried out with a heavy suspension of an actively growing tumor in sterile normal saline, 0.3 cc. being injected in one testicle. All animals of a group were inoculated at the same time.

The course of the disease was followed in each animal by frequent clinical examinations, special attention being paid first to the time of initiation of the growth of the primary tumor, its general rate of growth, and its ultimate condition, that is, continued growth, regression, or healing; second, the time of appearance, the location, and the course of metastases in superficial parts of the body; and third, the general physical condition including body weight determinations. The experiments were discontinued 8 to 9 weeks after inoculation, at which time all surviving animals were killed by an injection of air into the marginal ear vein. The same procedure was carried out in the case of any animal whose condition became critical before the end of the observation period. A few rabbits developed "snuffles" while under observation, but in the present analysis of results, they have not been separately considered.

The character of the disease was further appraised by the postmortem examination findings of each rabbit. The site of any macroscopic tumor growth was noted and in addition, such features as size, destructiveness, and approximate amount of living and necrotic tumor tissue were described. In the case of those rabbits in which tumor was found at the end of the experimental period, a classification of probable death or probable recovery was made, based upon the location and character of the tumor growths. The probable deaths, for example, include those instances in which both suprarenal glands or the hypophysis were involved by apparently living tumor while the probable recoveries include cases in which only necrotic and evidently healing tumors were found in such locations as the retroperitoneal lymph nodes. This classification is based upon the results of a 10 years' experience with this tumor.

The general conduct of the experiments and the technique employed in the blood examinations were similar to those carried out on normal rabbits (1). Suffice it to say here that a variable number of examinations were made during the 3 weeks preceding inoculation and once a week thereafter during the postinoculation period of 2 months. In the case of Groups II and VII, the examinations were made more frequently; in the present analysis of results, these observations have been averaged for each week. The red and white blood cell counts were made with standardized pipettes; the hemoglobin determinations were carried out with a Newcomer hemoglobinometer. Differential white blood cell counts were made with the supravitral neutral red technique, 100 cells being counted in each preparation.

In the present consideration of results, each animal has been allocated to one of the four following groups depending upon the postmortem findings: deaths, probable deaths, probable recoveries, and recoveries. The findings of each weekly blood examination of each individual animal in these groups were calculated in terms of

In the first place, it must be remembered that the experiments were carried out in different years and in different seasons of the same year. It is to be expected, therefore, that some spontaneous variations in the blood picture might occur as in the case of normal rabbits observed over similar periods of time (2, 3, 4, 5). Such variations, which may be attributable to general environmental conditions as well as the occasional irregular values of individual animals, are minimized by the group method employed for the analysis of the results. Secondly, it should be pointed out that the course of this neoplastic disease is subject to considerable variation, not only as regards individual animals of the same series, but in different series as a whole. These variations are shown by such features as the rate of growth of the primary and metastatic tumors and the death rate, the time at which a fatal outcome occurs, the incidence, distribution, and extent of metastases, and the number of recovered animals. In some series, the degree of malignancy is high while in others it is low and in still others, it lies between these extremes. In the present experiments, as shown in Table II, the varying numbers of deaths, of probable malignancy presented by the animals of these experiments. It is evident that the combination of individual animal observations derived from successive weekly examinations is subject to correction on the basis of the course of the disease in each particular animal. It would be difficult to follow this procedure, however, because the only available features of the disease which lend themselves to a practical method of appraisal are, first, the primary tumor, the development and growth of which is not necessarily an index of tumor growth elsewhere, and second, the occurrence of metastases in superficial parts of the body. The relatively low incidence of such superficial tumors except in cases of evident high malignancy, precludes the use of this feature. It has been found by experience, however, that gross changes in tumor growth are usually evident from week to week, and hence this time interval was used for the present observations. The combination of the results of individual animals from different series has been made on the same time basis as affording the least opportunity for error.

In the third place, the results have been considered from the standpoint of the mean values of a group rather than from that of the individual animal since exaggerated or unusual findings in particular rabbits as well as the results of technical errors of blood examination are thereby minimized. While the numbers of rabbits in each group are not large, they are sufficiently comparable to permit group comparisons, that is, 25, 16, 22, and 15 animals in the deaths, the probable deaths, the probable recoveries, and the recoveries, respectively. Finally, it must be remembered in comparing the results of the four groups that in the case of the fatal group, its numbers became progressively decreased and the reliability of the mean values is thereby lessened. 5 weeks after inoculation there were 24 animals; at the 6th week, 17; at the 7th week, 10, while there were but 3 at the 8th week. It should also be remembered with respect to the rabbits in the group of probable recoveries that all showed some evidence of tumor at postmortem

in Groups III, VI, VIII, and IX and two animals in Group VII.² Metastases in such locations as the skin, the eyes, and the superficial lymph nodes were observed clinically in several rabbits and there were a number of cases in which paralysis of the hind quarters developed, due to metastatic growths in the spine. In the case of the recoveries and the probable recoveries, an excellent physical condition was maintained. This was also true in certain of the animals classified as probable deaths but not in others, particularly in those cases in which metastatic involvement of the jaws occurred. With respect to those rabbits which died from the disease, the physical condition was affected usually during the week preceding the fatal outcome.

Table II shows the distribution of animal material of the nine experiments; the sole criterion of this distribution, it may be pointed out again, was the character of the disease.

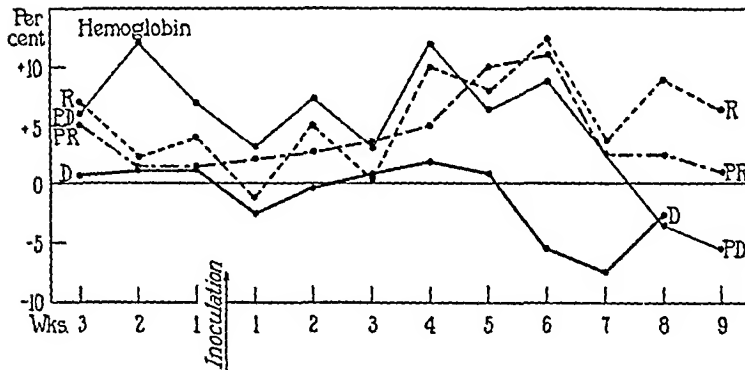
TABLE II

Group	No. of rabbits	Deaths	Probable deaths	Probable recoveries	Recoveries
I	9	5	2	0	2
II	5	2	0	2	1
III	9	3	4	1	1
IV	9	3	1	2	3
V	10	2	1	2	5
VI	4	2	0	1	1
VII	12	5	3	4	0
VIII	12	2	4	5	1
IX	8	1	1	5	1
Total.....	78	25	16	22	15

DISCUSSION

There are certain general features of these experiments which should be referred to before discussing the results.

² It should be mentioned in this connection that tumor growths in other parts of the body may be found in cases in which a primary tumor fails to develop after intratesticular inoculation. Among the present six rabbits in which the primary tumor did not develop, four showed tumors elsewhere at postmortem examination 2 months after inoculation. The two rabbits in which no tumor was found have been classified as recoveries; there may have been metastatic growths which had regressed and healed.



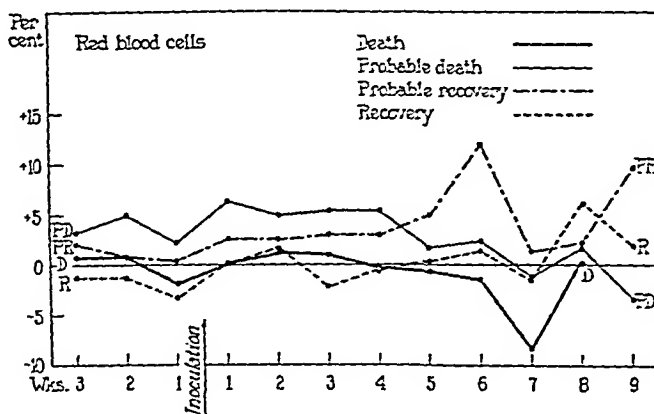
TEXT-FIG. 2. Hemoglobin content

Red Blood Cells and Hemoglobin:—The curves representing the mean levels of the red blood cells during the 3 weeks prior to inoculation (Text-fig. 1) show a range from 4 per cent above the standard value of 5,200,000 cells per c.mm. in the case of the probable deaths, to 3 per cent below this value in the recovered group; the curves representing the deaths and the probable recoveries lie between these limits. For the two first observations after inoculation, the red cell values became increased in all four groups as is shown by the upward trend of the curves. In the case of the fatal group, the curve then describes a gradual fall for 4 weeks, with an abrupt and marked drop to minus 7 per cent at the 7th week and an equally abrupt terminal rise to the base line. It will be remembered that the last two examinations present but 10 and 3 animals; the rise of the last observation was caused by the results on one rabbit, the last values of which, however, were much lower than the preceding ones. The curve of the probable deaths is maintained at approximately a plus 5 per cent level through the 4th week; it then descends in a fairly orderly fashion to a final level of 3 per cent below the base line. The findings in the probable recoveries and the recoveries were more irregular than those of the other groups, but there was a definite tendency toward progressively higher values, particularly in the last four observations; the curves for both groups end well above the base line.

The mean values of the hemoglobin content were, with the exception of the fatal group, more irregular than those of the red blood cells. This feature is brought out by comparing the curves of Text-figs. 1 and 2; it will be seen at once that in the curves representing the hemo-

examination. In many instances, the growths appeared to be largely or entirely necrotic and in all cases what tumor there was, either living or necrotic, was in a location which presumably would not have led to the death of the animal; had the experiments continued, it was felt that ultimate regression and healing would have occurred.³ In six instances, the residual tumor comprised only necrotic nodules in the inoculated testicle.

The results obtained will now be discussed, beginning with the erythrocytes and the hemoglobin content and then taking up the total white count and the various classes of white cells. The most striking changes were observed in the fatal group as might be expected, and therefore, these results will first be considered; the recovered group, on the other hand, showed comparatively slight alterations.

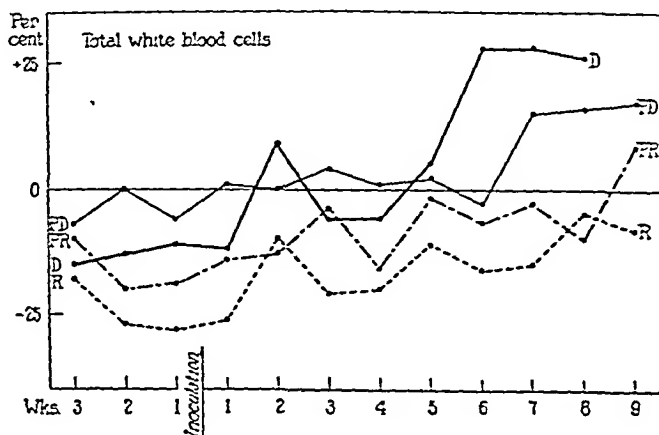


TEXT-FIG. 1. Red blood cells. In this and subsequent charts, the values for the four animal groups are expressed in the form of mean percentage deviations (algebraic sum) from standard values.

³ In this connection, it should be mentioned that it has not been possible to reinoculate recovered tumor rabbits by any of the usual routes, such as the intravenous, the intracutaneous, or the intracerebral. The only site which so far has proven successful has been the suprarenal gland (unpublished experiments of Pearce and Van Allen); other tissues appear to be immune (11). It appears extremely unlikely that both suprarenals will eventually become involved when the only tumor present 2 months after inoculation is confined to the residual primary tumor or to growths in such comparatively remote sites as the eye, the connective tissue, or the retroperitoneal lymph nodes. It is also unlikely, even if one suprarenal is the seat of a metastasis, that the second will become involved after 2 months of the disease. All such cases are classified as probable recoveries.

standard value of 9560 cells per c.mm.; the weekly variations of any one group, however, did not exceed 10 per cent of this value (Text-fig. 3). After inoculation, the trend in all groups was toward increased values throughout the period of observations as is shown by the upward direction of the curves. This trend was most marked in the case of the fatal group in which the highest levels were found during the last 3 weeks; the curve shows that a value of 25 per cent above the standard value was reached as compared with the average minus 15 per cent value of the preinoculation period. The curve representing the probable deaths shows a similar although slightly less marked change and in addition, it will be noted that the time of the rise occurred later. In the case of the probable recoveries, the findings were more irregular than with the deaths or the probable deaths, but as the general direction of the curve indicates, there was a similar tendency toward increased white counts which, however, were not as high as with either of the other groups. In the recovered animals, the total white cells showed a slight numerical increase after inoculation amounting approximately to 15 per cent above the preinoculation level. It will be noted, however, that at no observation did the total white count of the recovered group exceed the standard value while this was found to be the case with the other groups, particularly with the deaths and the probable deaths.

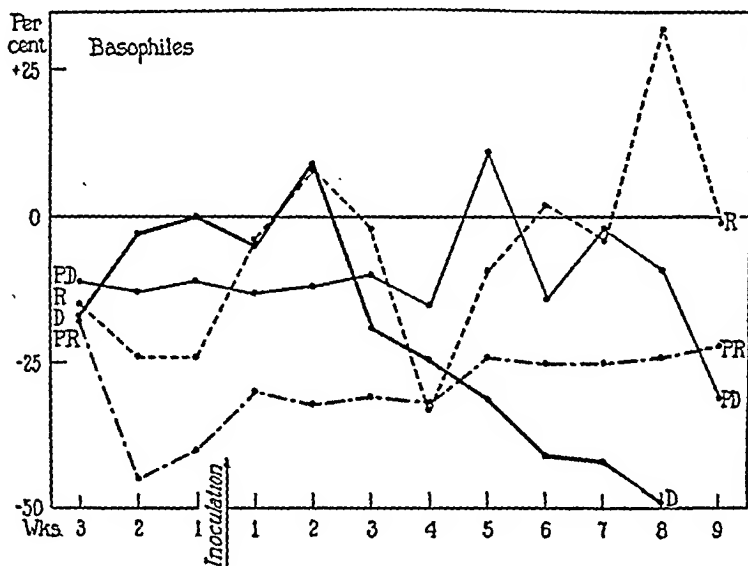
globin content, the excursions are more abrupt and the changes in level more pronounced. Before inoculation all four curves are above the base line which represents a value of 63 per cent; that of the probable death group contains one marked irregularity. After inoculation, three curves show an immediate drop followed by a rise. In the case of the deaths, a fairly constant level in the neighborhood of the base line is maintained for 5 weeks; during the next 3 weeks, the curve falls to 5, 7.5, and 2.5 per cent below the normal value. In the first half of the postinoculation period, the curve of the probable death group ranges between 3.5 and 7 per cent above the base line but during the last half, it falls steadily from this high level to a minus 5.5 per cent, a total change of 12.5 per cent. Despite irregularities, the curve of the recoveries shows a tendency toward the maintenance of higher values after inoculation. A similar tendency is seen in the probable recoveries until the last two observations when the direction of the curve appears to be definitely downward. It should be noted, however, that the values of both the probable recoveries and the recoveries are above the base line throughout the observation period while those of the deaths and probable deaths fall below the normal value during the latter part of this period.



TEXT-FIG. 3. Total white blood cells

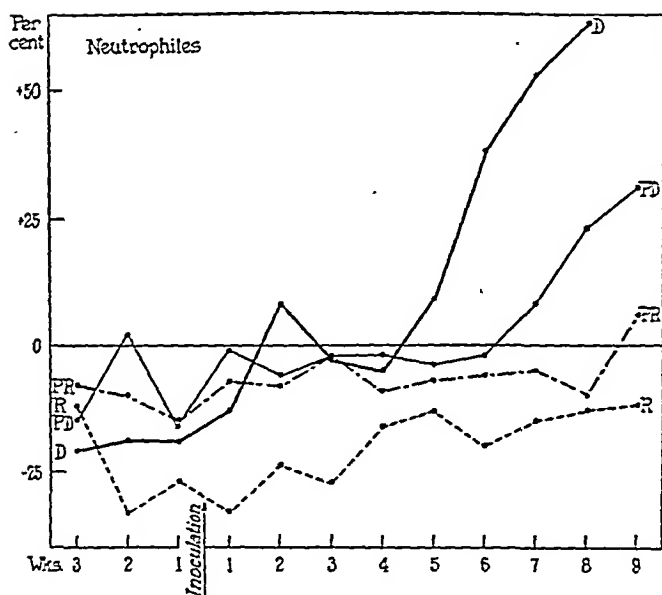
White Blood Cells:—Before inoculation, the mean total white counts of all four groups of animals varied from 5 to 25 per cent below the

at the conclusion of the experiment. With the complete recoveries, on the other hand, the curve throughout lies below the base line.



TEXT-FIG. 5. Basophiles

Basophiles:—The observations with respect to the basophiles are illustrated by the curves in Text-fig. 5. It will be seen by referring to these curves that there was considerable variation in the mean group levels of these cells during the 3 weeks prior to inoculation, the general range being from 5 to 25 per cent below the standard value of 950 cells per c.mm. In the groups of deaths and probable recoveries, moreover, the findings were comparatively irregular, the shifts in level amounting approximately to 15 and 25 per cent respectively. After inoculation it was found that in the case of the fatal group, the number of basophiles became markedly decreased, the change being pronounced at the 3rd week and continuing thereafter throughout the observation period; at the last examination, the count had fallen to 50 per cent below the standard value. With the probable deaths, a somewhat similar change was found after the 5th week of the disease, but it was less marked and less consistently followed as will be seen by comparing the respective curves. The last observation of the group of probable deaths was 30 per cent below the standard value as compared with the high point of plus 10 per cent at the 5th week and a



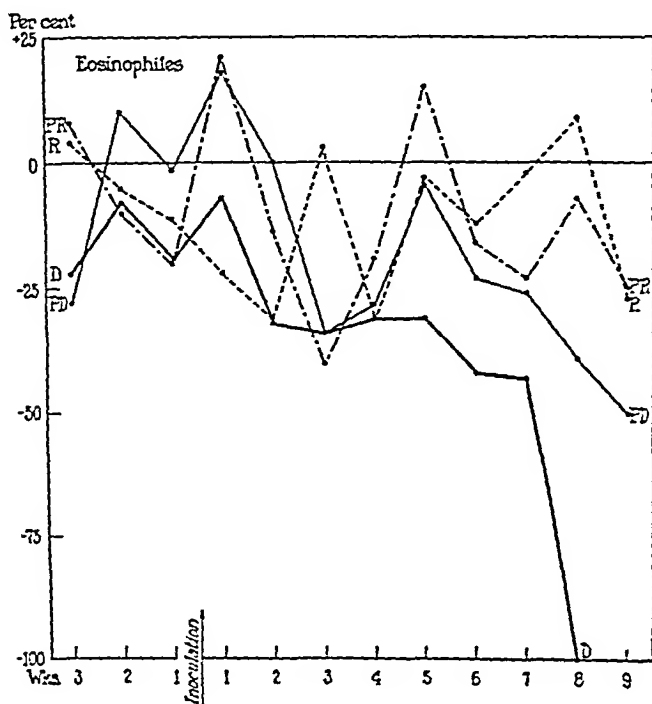
TEXT-FIG. 4. Neutrophiles (pseudo-eosinophiles)

Neutrophiles (Pseudo-Eosinophiles):—The preinoculation levels of the neutrophilic leucocytes in all four groups were below the standard value of 4340 cells per c.mm., the general group range being minus 10 to 25 per cent of this value (Text-fig. 4). The postinoculation observations showed that in the fatal group, the neutrophiles became increased, that this change became pronounced in the 5th week, and that a figure of 65 per cent above the standard value was reached at the time of the last examination. This value represents a rise of 85 per cent above the preinoculation level of the group. A similar but less marked increase to 30 per cent above the standard value occurred in the group of probable deaths and as will be seen by referring to the curves in Text-fig. 4, it occurred 2 weeks later than the decided rise of the neutrophiles of the fatal group. The curves representing the probable recoveries and the recoveries show that while there was also a tendency toward increased neutrophile counts after inoculation, the changes were comparatively slight. It is of interest to note, however, that the curve of the probable recoveries ends above the base line as was the case with the other two groups in which tumor was present

Eosinophiles:—The mean preinoculation eosinophile counts of all four groups were irregular. The limits of the irregularities as shown by the curves in Text-fig. 6 were 10.2 per cent above to 25.5 per cent below the standard value of 215 cells per c.mm.; the widest range of weekly variation of individual groups was 15 per cent of the standard value except in the case of the probable recoveries in which it was 35.5 per cent. After inoculation, the irregularities were generally more pronounced, a feature which is brought out by the pronounced swings of the curves. With the fatal group, there was a definite drop in the mean number of eosinophiles at the 2nd week; this new level was continued for three observations and was succeeded by even lower levels, and eventually at the last examination, no eosinophiles were found. The results in the other groups were not so clean-cut. In the probable deaths a similar but less pronounced decrease of eosinophiles occurred as shown by the general downward trend of the curve from its highest point in the 1st week after inoculation, although there is an upward swing during the 4th and 5th weeks corresponding to the stabilized period of low levels in the fatal group. With the probable recoveries and the recoveries, the results are not striking. It will be noted, however, that after the 4th week, the curves for these two groups are consistently higher than those of the deaths and the probable deaths, and that for the last four observations the mean numbers of eosinophiles in the recoveries were greater than in the probable recoveries. These features taken in conjunction with those relating to decreased values in the groups of deaths and probable deaths suggest that an increased eosinophile count is probably characteristic of the recovered state.

general preinoculation level of minus 10 per cent. In contrast with these findings, a sustained drop in the mean basophile count of the probable recoveries and recoveries was not observed. The curve representing the recovered group shows two major swings of considerable amplitude, but during the latter half of the observation period, that is, after the 4th week, the general trend is definitely upward and it ends on the base line. In the case of the probable recoveries, although the direction of the curve is slightly upward, it is maintained fairly constantly at levels of 30 and 25 per cent below the standard value.

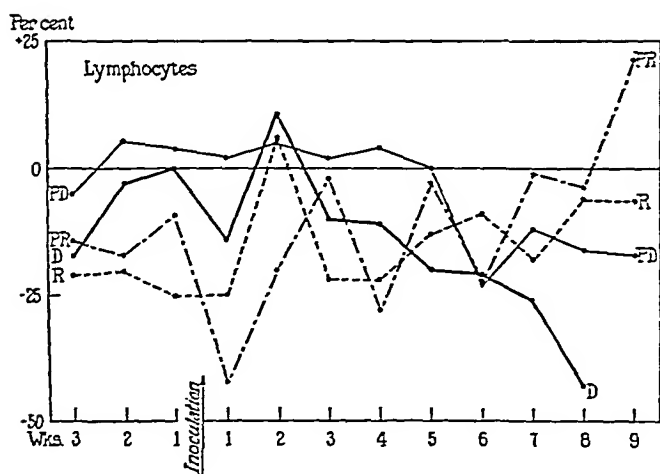
It will be noted that during the latter half of the observation period, the basophile curves of the three groups of animals in which tumor was found at postmortem examination, all lie below the standard value while that of the recovered group in which no tumor was found lies at or above this value.



TEXT-FIG. 6. Eosinophiles

was a moderate decrease in the mean numbers of lymphocytes which persisted to the end of the observation period. The extent of the decrease amounted to approximately 20 per cent on the present scale as is shown by the curve in Text-fig. 7. The results in the probable recoveries and the recoveries are less definite. It will be seen, however, by referring to the curve representing the probable recoveries that despite frequent and comparatively wide variations, there was a distinct tendency in the latter half of the observation period for the lymphocytes to become increased over the average level of the pre-inoculation period and of the values found in the first weeks after inoculation. The curve rises from a point 25 per cent below the standard value at the 4th and 6th weeks after inoculation to a final level of plus 20 per cent. With the recovered group, the curve indicates that following the low levels of the 3rd and 4th weeks after inoculation, there was a fairly consistent trend in the direction of slightly increased lymphocyte counts which were still maintained at the last examination.

It would appear from these results that the mean number of lymphocytes in the peripheral blood tends to be somewhat decreased in rabbits succumbing to the effects of this neoplastic disease and that a similar but less marked effect is associated with a less severe process. On the other hand, the mean lymphocyte values tend to be slightly increased in rabbits in which the process is mild or in which recovery takes place.



TEXT-FIG. 7. Lymphocytes

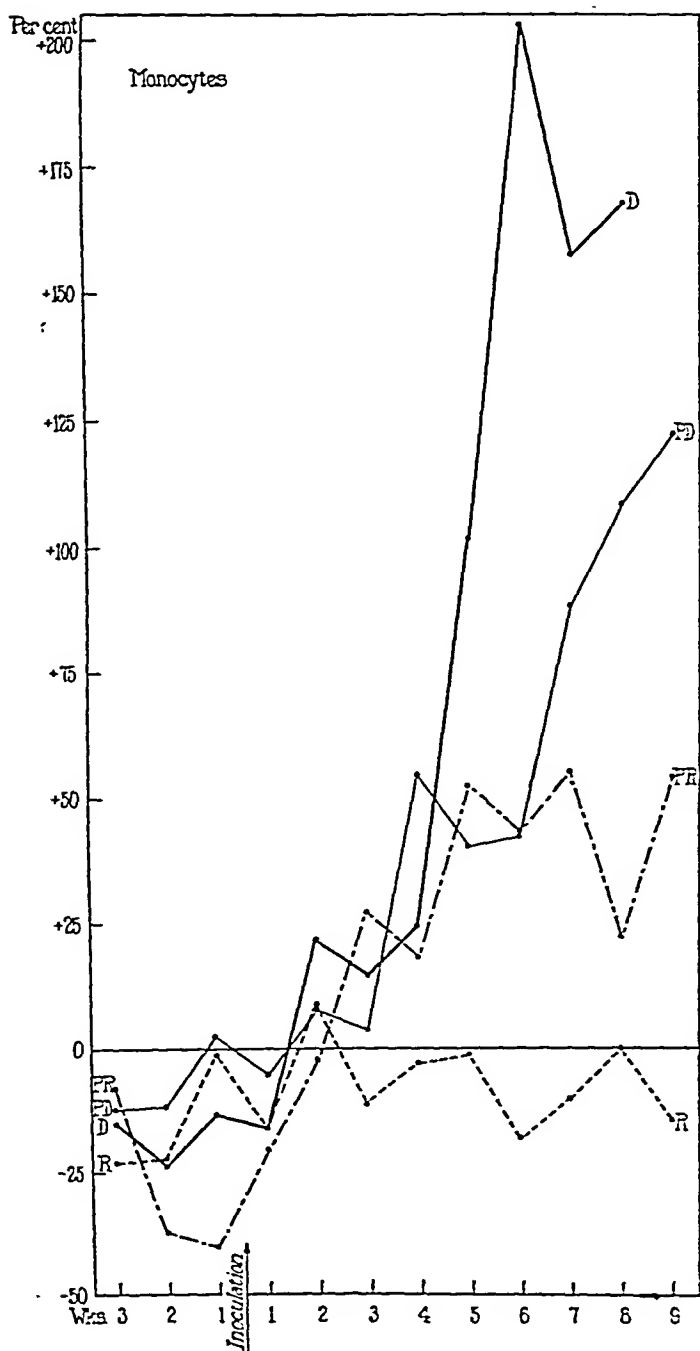
Lymphocytes.—The observations regarding the lymphocytes are illustrated by the curves in Text-fig. 7. It will be seen by referring to these curves that the average mean levels of the lymphocytes in the four groups of rabbits before inoculation were variable, their limits lying between the standard value of 3050 cells per c.mm. in the case of the probable deaths, to approximately 20 per cent below this figure in the case of the recovered group. Irregularities of individual groups ranged from 5 to 15.5 per cent on the standard value scale.

A week after inoculation, a drop in the mean numbers of lymphocytes was observed in two groups, the deaths and the probable recoveries, while in the others, little or no change was found; at the next observation, 2 weeks after inoculation, the numbers in three groups were considerably increased. From this time onward, the lymphocytes of the fatal group showed a steady numerical decline which is well brought out by the curve of Text-fig. 7; at the final observation, the level reached the low point of 40.5 per cent below the standard value as compared with an average preinoculation value of 5 per cent below and with 10 per cent above the standard value at the 2nd week after inoculation. In the case of the probable deaths, there was no significant change until the 6th week after inoculation. At this time, there

Monocytes:—The observations on the monocytes are illustrated by the curves in Text-fig. 8. The preinoculation levels in all four animal groups were lower than the standard value of 1000 cells per c.mm. In the case of the deaths, the probable deaths, and the recoveries, successive weekly values showed variations of 10 to 20 per cent of the standard value; with the probable recoveries, the range extended to 30 per cent. The average levels in this period, as may be seen by the curves, were approximately 20, 7, 14, and 27 per cent below the standard value for the deaths, the probable deaths, the probable recoveries, and the recoveries respectively.

After inoculation, the monocytes in all groups except the recoveries, became markedly increased, that is, a peripheral blood monocytois was associated with tumor growth. Beginning at the 2nd week, all four groups showed increased mean numbers of monocytes, the levels attained being higher than the standard value in three groups and equal to it in the fourth. In the case of the fatal group, this higher level was maintained for the following 2 weeks, and from this time to the end of the observation period, very large numbers of monocytes were found. 6 weeks after inoculation, the increase amounted to 200 per cent above the standard value; in the 7th and 8th weeks the figures were slightly smaller, but it will be remembered that these last two observations represent 10 and 3 animals (the most resistant of the group) as compared with 17 at the 6th week. The findings with respect to the group of probable deaths were comparable to those of the fatal group as is shown by the respective curves. It will be noted, however, that the time of the first marked monocytic increase occurred later, that is, at the 7th as compared with the 5th week in the fatal group, and furthermore, that the magnitude eventually attained at the end of the observation period was not as great, that is, 125 as compared with 170 per cent. The findings of the last three observations in the probable death group show progressively larger numbers of monocytes as is illustrated by the regularly rising curve, and this feature is undoubtedly influenced by the fact that each observation represents the same number of animals, none dying before the end of the experiment as was the case with the fatal group.

In the probable recoveries, increased numbers of monocytes were observed to the extent of 50 per cent above the standard value. This



TEXT-FIG. 8. Monocytes

The time of these changes should be referred to. In the fatal group, the curves representing the red blood cells, the hemoglobin content, the various classes of white cells, and the total white count show a decided trend of direction at the 4th and 5th weeks after inoculation although the trend itself may have been initiated earlier, as was the case with the monocytes. It will be noted in the group of probable deaths that, on the whole, the changes which were of the same general character as those of the fatal group, occurred somewhat later. This finding is in keeping with the differences in malignancy level of the two groups. In this connection, it should be mentioned that although the rate of growth of the primary tumor and the distribution and growth of metastatic tumors vary widely, postmortem examination of a large number of rabbits at variable periods of the disease has shown that by the 3rd or 4th week after inoculation, the tumor process is well established in cases of pronounced or average malignancy.

As far as the probable recoveries and recoveries are concerned, the results in general correspond to what might be expected in the light of the findings in the groups characterized by an actively progressing tumor process. Thus, in the case of the monocytes which is the most striking example, increased counts of a moderate degree were observed in the group of probable recoveries, but there was little change in the group of recoveries.

The differences in the blood picture of rabbits representing the two extremes of reaction to the malignant disease, that is, the fatal and the recovered cases, are brought out by the accompanying graphs in which the last observations of these groups are illustrated (Text-fig. 9). For the recovered group, the values are, of course, those given in the curves of the text-figures; for the fatal group, on the other hand, the values are different since they represent the combined last observations of each animal irrespective of the time of death.

condition was attained by the 5th week after inoculation and corresponds to a similar level on the part of the probable death group and to a value of plus 100 per cent for the fatal group. During the last four observations, the curve representing the probable recoveries is irregular but on the whole, it tends to remain at the level of 50 per cent above the standard value. In the case of the recovered animals, the highest monocyte level observed was at the 2nd week after inoculation, that is, 9 per cent above the standard value. At subsequent examinations to the end of the observation period, there was an irregular decrease in the mean numbers of monocytes, and it will be noted that after the 2nd week, the curve does not again rise above the base line and that its general contour resembles its preinoculation portion.

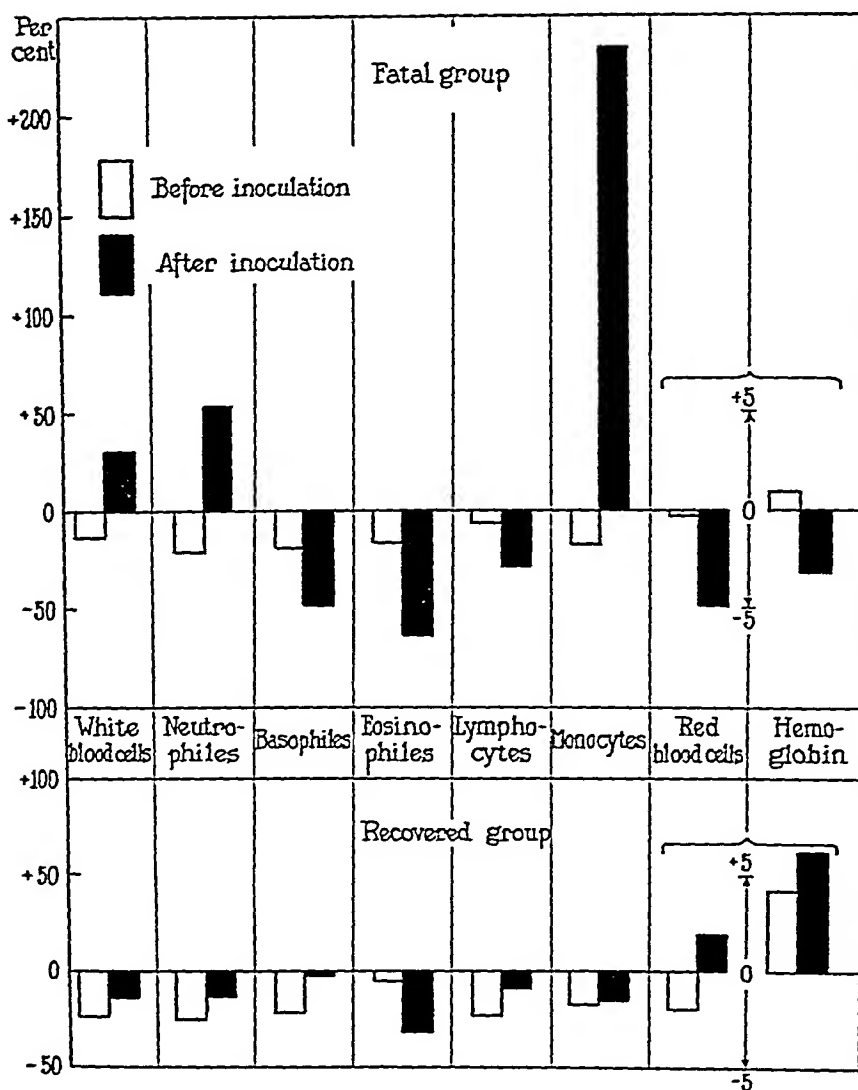
The above discussion of these results may be summarized as follows: During the course of this particular neoplastic disease, certain alterations of the peripheral blood picture were observed. The most striking changes during the course of a fatal or probably fatal condition concern the monocytes, a pronounced numerical increase of which was found, and secondly, the eosinophiles, the numbers of which were markedly reduced. In the case of the basophiles, a change similar to but less marked than that of the eosinophiles was observed (12). The relation of the numbers of lymphocytes to the course and type of disease was less definite than that of the monocytes, eosinophiles, and basophiles, but the findings showed a general correspondence to those of the eosinophiles and basophiles rather than to the monocytes. In the case of the neutrophiles, it was found that their numbers became increased in an actively progressing disease. As would be expected, the character of the total white count was greatly influenced by the neutrophiles. The very large numbers of monocytes in the fatal and the probable death groups also contributed to the increased total white counts. And finally, in conditions of pronounced or well marked malignancy, decreased numbers of red cells and a lowered hemoglobin content were observed; the change in the red cells preceded that of the hemoglobin.⁴

⁴ In connection with these results in cases of well marked malignancy, it is of interest to recall that the blood count of the original tumor rabbit taken shortly before death showed the characteristic findings here described (10).

The outstanding changes which have been noted in the present experiments far exceed the spontaneous variations found in groups of normal rabbits examined for comparable periods of time (2, 3, 4, 5). Strictly speaking, the present results cannot be compared with those of the normal animals because they are derived from an analysis in which the findings of individual rabbits from different groups are combined, while with the normal animals the entity of each group was preserved. It may be stated, however, that an analysis of each animal group of these experiments has been made and that the results are substantially the same as those presented in this paper.

It should be mentioned that all the characteristic blood picture changes associated with the malignant process as determined by the analysis of the material on a combined group basis, were not invariably found in individual animals; in certain cases, one or more of these changes were present while others were lacking. These instances were rare, however, when one takes into consideration the general character of the blood counts over the entire observation period. There was one striking example of a fatal case with a widespread distribution of tumor in which the monocytes and neutrophils were not increased, the lymphocytes were unchanged, and the basophils were only slightly diminished; there was, however, a decreased red count, a lowered hemoglobin content, and a decreased eosinophile count.

At the present time, no interpretation of the results of these experiments has been made. It is evident that in rabbits in which active and extensive tumor growth takes place, that is, in animals with a low resistance to the disease, the numbers of peripheral basophils, eosinophils, and probably the lymphocytes are low in contradistinction to the high numbers of monocytes and neutrophils. But whether these states are the result of the tumor process or are more intimately connected with the cause of its activity, cannot now be determined. The results of the tissue studies carried out in connection with observations on the peripheral blood as well as those bearing on the character of the blood picture before inoculation may throw some light on the subject.



TEXT-FIG. 9. Last observations on the fatal and recovered groups. Values are expressed in the form of mean percentage deviations (algebraic sum) from standard values.

SUMMARY AND CONCLUSIONS

Successive blood counts at weekly intervals were made on rabbits inoculated with a transplantable malignant neoplasm of epithelial origin. There were 78 animals distributed in 9 groups; the period of observation after inoculation was 2 months. The results have been considered with respect to the character of the tumor process as determined by postmortem examination, the animals being classified as deaths, probable deaths, probable recoveries, and recoveries. The blood findings have been analyzed on the basis of the values derived from normal rabbits. A comparison has also been made with the results of preinoculation counts.

In rabbits in which the tumor process was of pronounced or well marked severity, the numbers of monocytes became greatly increased; the neutrophiles and the total white blood cells were also increased; the eosinophiles, and to a less extent, the basophiles and lymphocytes were decreased; the red cell count was decreased and the hemoglobin content was lowered.

In rabbits which recovered from the tumor inoculation, there were no outstanding changes in the blood picture when the findings for the entire observation period were considered. In the group of probable recoveries, the results were variable; there was, however, a definite although moderate increase of monocytes, and in general, the findings reflected the mildness of the disease when compared with those of the fatal cases and the group of probable deaths.

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IRON IN TUBERCULOUS AREAS

EXPERIMENTAL

Bovine tuberculosis was induced in rabbits by the intravenous injection of either 0.01 mg. or 0.001 mg. of a saline suspension of Ravenel strain. Several weeks later a large number of these animals were killed by ether anesthesia. The lungs which showed, as a rule, extensive tuberculosis were carefully removed and dipped for about an hour in acidified potassium ferrocyanide (3 parts of 1 per cent HCl to one part of 2 per cent $K_4Fe(CN)_6$).

TABLE I
Presence of Iron in Tubercles of Non-Injected Animals

Rabbit No.	Interval between injection of tubercle bacilli and death of animal <i>days</i>	Prussian blue reaction in tubercles of lung
1*		
2*	14	
3*	19	0
4*	19	0
5	20	0
6**	22	0
7**	25	0
8*	28	0
9	35	0
10***	36	Trace
11	40	Trace in 2 or 3 tubercles
12	52	0
13	52	0
14	52	0
15	53	0
	53	0

* These rabbits received 0.01 mg. of bovine Ravenel strain. All the others received 0.001 mg.

** These two rabbits each received a single injection of 20 cc. 0.25 per cent ferric chloride.

*** This rabbit was allowed to die; the lungs were tested for iron on the day of its death.

The results of the tests are shown in Table I. It is evident that in the vast majority of these animals the tubercles in the lungs exhibited a negative Prussian blue reaction. In this connection it is interesting to note that many years ago Gierke (7) pointed out that calcified tubercles in the lungs failed to react to the microchemical test for iron. It should, however, be mentioned that frequently when a

THE ACCUMULATION OF IRON IN TUBERCULOUS AREAS

BY VALY MENKIN, M.D., AND MIRIAM F. MENKIN

(From the Department of Pathology, Harvard University Medical School, Boston)

PLATES 37 AND 38

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In previous communications (1, 2, 3) it has been shown that a vital dye, trypan blue, or iron in the form of its salt, ferric chloride, when injected into the circulating blood stream rapidly accumulates in an area of inflammation, where the substance is fixed and fails to drain to the tributary lymph nodes.

Bowman, Winternitz, and Evans by microscopic studies (4) found that, in experimental tuberculosis, trypan blue injected intravenously stains tubercles in the liver. They pointed out the great affinity of giant and epithelioid cells for this vital dye, which was always found as granules within these cells. Lewis (5) showed that if the cornea of a rabbit is inoculated with a living culture of the tubercle bacillus, a progressive lesion results characterized by an intense congestion of the conjunctiva. If the animal is given an intravenous injection of trypan red 24 hours or more after such inoculation, the fluid in the anterior chamber of the inoculated eye always becomes colored. We have been able to confirm these earlier studies by gross examination. Trypan blue injected in the circulating blood stream of rabbits rapidly accumulates in tuberculous areas of the lung.

In view of the observation that iron, intravenously injected, accumulates in inflamed areas, experiments were set up to determine whether this metal when injected into the circulating blood stream would accumulate and be detectable in tubercles. In a preliminary report by one of us from The Henry Phipps Institute (6) it was shown in a few experiments that after several intravenous injections of ferric chloride an accumulation of iron within the caseous areas of tubercles in lungs was readily demonstrable by a qualitative test (Prussian blue reaction). These studies have been continued and considerably extended so as to include quantitative determinations on the accumulation of this metal in tuberculous areas.

per cent solution of ferric chloride produced no demonstrable Prussian blue reaction in the tubercles (Rabbits 6 and 7, Table I). Two injections of the ferric salt likewise produced essentially a negative Prussian blue reaction in Rabbit 18 (Table II). Several intravenous injections of the ferric salt at least are necessary before a positive Prussian blue reaction can be elicited in the tubercles of the lung.

The Prussian blue reaction involves the tubercles on the surface of the lung since these are the only ones in direct contact with the potassium

TABLE II

Presence of Iron in Tubercles of Animals after a Number of Intravenous Injections of Ferric Chloride

Rabbit No.	No. of 5 cc. injections of 0.25 per cent ferric chloride	Interval between injection of tubercle bacilli and death of animal	Prussian blue reaction in tubercles of lung
		days	
18	2	22	Trace in few tubercles
19	3	24	+
20*	16	37	+
21	9	38	+
22	4	39	+
23	2	42	+
24	7	43	+
25**	22	84	+
26**	19	87	+

* Received 0.01 mg. bovine Ravenel strain. All other rabbits received 0.001 mg.

** These rabbits were allowed to die of their disease; the lungs were tested for iron on the day of death.

ferrocyanide solution. The deeper layers of caseating tissue consequently do not reveal the presence of the metal by the qualitative test. ✓

Histological studies of lung tissue of animals injected intravenously several times with ferric chloride and tested for the presence of the iron by the Prussian blue reaction show the metal to exist chiefly within the caseous centers of the tubercles (See Fig. 2 and Table II, Rabbit 26). Fig. 2 shows that the intense blue reaction (shown on the figure in black) is not distributed homogeneously throughout the caseous area. The figure is a low power reproduction; careful studies under higher magnification show that the granules sur-

rabbit was allowed to die spontaneously of tuberculosis and then placed on ice for a day or two before removing the lungs, the latter would reveal a distinct positive Prussian blue reaction within the caseous areas of tubercles. Evidently such areas contain a considerable quantity of iron bound up in a form that does not give the Prussian blue reaction prior to some degree of postmortem change. For this reason the qualitative test was always made on the lungs of an animal immediately after killing it, or, when the animal died spontaneously, on the day of death (Rabbit 10, Table I).

The experimental rabbits received an intravenous injection of either 0.01 or 0.001 mg. of saline suspension of bovine tubercle bacilli (Ravenel strain). From 3 to 5 weeks later these animals were started on a course of daily intravenous injections each of 5 cc. of a 0.25 per cent solution of ferric chloride crystals. The solution was usually heated slightly and injected very slowly. The number of injections varied a great deal. This material proved injurious when it escaped into the tissue about the marginal vein of the ear. After numerous daily injections, the development of thrombi in vessels and of an extensive local inflammatory reaction often prevented further administration of the iron salt. The rabbits as a rule were killed after several injections of the ferric salt solution. The lungs which showed extensive pulmonary tuberculosis were carefully removed and placed for about 1 hour in acidified potassium ferrocyanide.

Within a very short time, in some cases amounting to only a few minutes, the tubercles exhibited a marked Prussian blue reaction on gross examination. The blue color was intense in the caseous or central part of the tubercle (See Fig. 1 and Table II, Rabbit 20). The result was very different from that in ordinary inflamed areas. Dye or iron which penetrates into the periphery of these by way of the blood stream fails to enter the central part in which the circulation is relatively inactive (1, 2). It is to be noted in this connection that various dyes that cannot penetrate living cells are able to stain dead or dying cells (8).

The results of all the experiments are shown in Table II. Both Rabbits 25 and 26 lived for 13 days after the injections of ferric chloride had been discontinued. These two animals died of their tuberculosis and the lungs were tested on the day of death. In both of these rabbits an intense blue reaction in the caseous areas was obtained a few minutes after dipping the lungs into acidified potassium ferrocyanide solution. It was found that a single injection of 20 cc. of 0.25

16 hours at a temperature ranging from 120°C. to 130°C. The lungs of tuberculous animals that had received no iron were treated in the same manner, to serve as controls. Both sets of animals had received the same dose of tubercle bacilli intravenously (0.001 mg. of a bovine Ravenel strain, with the exceptions of control Rabbit 8 and experimental Rabbit 20, each of which received 0.01 mg.).

Quantitative determinations of iron in tissue were performed according to the method of Kennedy (9) with only slight modifications. 20 cc. of concentrated sulfuric acid and 17 cc. of 60 per cent solution of perchloric acid were added to a carefully weighed portion of dried lung in a Kjeldahl flask. The mixture was heated over a low flame for about 10 minutes until complete digestion took place

TABLE III

Iron Content of Tuberculous Lungs in Animals Injected Daily with Ferric Chloride and in Non-Injected Animals*

Non-injected animals			Injected animals			
Rabbit No.	Interval between injection of tubercle bacilli and death of animal	Iron content*	Rabbit No.	No. of 5 cc. injections of 0.25 per cent ferric chloride	Interval between injection of tubercle bacilli and death of animal	Iron content*
	days				days	
8**	35	55.5	20**	16	37	165.3
9	36	120.0	21	9	38	185.2
14	53	118.3	24	7	43	289.5
16**	66	163.6	25***	22	84	876.1
17***	67	123.6	26***	19	87	686.9

* Figures are expressed in milligrams of iron per 100 gm. of dry tissue.

** Received 0.01 mg. of bovine strain of tubercle bacilli.

*** These animals were not killed but allowed to die of tuberculosis.

and the solution appeared almost colorless. While the mixture was still hot, 0.8 cc. of 30 per cent hydrogen peroxide was added and the liquid was then cooled and diluted to 100 cc. with distilled water. 10 cc. of this solution was pipetted into a 50 cc. stoppered cylinder to which were added 10 cc. of amylic alcohol and 5 cc. of 20 per cent sodium thiocyanate. The mixture was immediately shaken and the iron was extracted by the amylic alcohol layer. This layer was pipetted off into a colorimeter cup and compared with a standard treated in exactly the same manner.

The results of the analyses are shown in Table III. It is clear that the lungs of tuberculous animals that had received repeated intravenous injections of ferric chloride contain more iron than do the lungs of non-injected animals. Although the marked increase in iron con-

rounding the caseous area are not iron deposits but nuclei. There is no evidence that iron particles have been previously taken up by cells which have then migrated into the growing tubercle and have broken down there. On this assumption one would still expect to find at the periphery of the caseous area a great number of mononuclear phagocytes loaded with particles giving the test for iron. These were not found. Only rarely, here and there, were blue granules observed within the cytoplasm of mononuclear phagocytic cells. The iron seems therefore to be deposited primarily within the areas of caseation. There is no indication that proliferation of fibrous tissue in the lungs is greater in injected than in uninjected animals.

In several animals the injections of ferric chloride were started on the day following inoculation with 0.01 mg. of bovine tubercle bacilli. Daily injections of the salt were performed for about 2 weeks. The lungs of these animals were then studied and found studded with miliary tubercles. On gross examination these showed either no areas of caseation or at most very few of them. When testing for iron in such tubercles the Prussian blue reaction was negative. This constitutes another evidence that iron is apparently deposited only where caseous areas are present.

Quantitative Studies on the Accumulation of Iron in Tuberculous Areas

In a previous communication (2) it has been shown that in uninjected animals an old inflammatory lesion of at least 70 hours duration reveals the presence of iron by the qualitative test. This is probably due to the degradation, in the late stages of inflammation, of hemoglobin from red corpuscles phagocytosed by tissue macrophages. It has been pointed out above that when the lungs of an uninjected tuberculous animal were tested for the presence of iron a positive Prussian blue reaction in the caseous areas was frequently obtained if the test was made some time after death. Presumably tubercles as such contain a larger quantity of iron than normal lung parenchyma. For this reason it was thought advisable to substantiate the qualitative findings on the accumulation of iron in tuberculous areas by quantitative determinations.

Portions of the lungs of rabbits that had received daily intravenous injections of 5 cc. of 0.25 per cent ferric chloride solution were placed in a dry oven for about

age of iron after its injection takes place principally in the liver. This is in agreement with earlier work. Boycott and Price-Jones (11) showed that in rabbits infected with *Trypanosoma brucei* an anemia developed owing to the destruction of red cells. The iron of the destroyed hemoglobin was found stored in the liver and in the spleen. Muir and Dunn (12) demonstrated that in acute hemolytic anemia nearly all the iron from the destroyed hemoglobin was deposited in the liver, spleen, and kidneys.

The quantitative results obtained further corroborate the qualitative tests and show that an accumulation of iron in tuberculous areas follows repeated intravenous injections of ferric chloride.

These experiments suggest that the accumulation of iron in tuberculous areas may have clinical application. It is conceivable that iron or iron-containing substances by their accumulation in tuberculous areas may alter the character or course of development of the disease. Further experiments are being conducted to investigate this question.

CONCLUSIONS

Repeated daily intravenous injections of ferric chloride solution are followed by an accumulation of iron in tuberculous areas of lungs. The iron accumulates in the caseous areas of tubercles and is demonstrable by the Prussian blue reaction.

Quantitative determinations corroborate these results and show that the iron content of lung tissue in tuberculous animals injected with ferric chloride exceeds that in normal animals injected with this salt, as well as that in non-injected tuberculous animals.

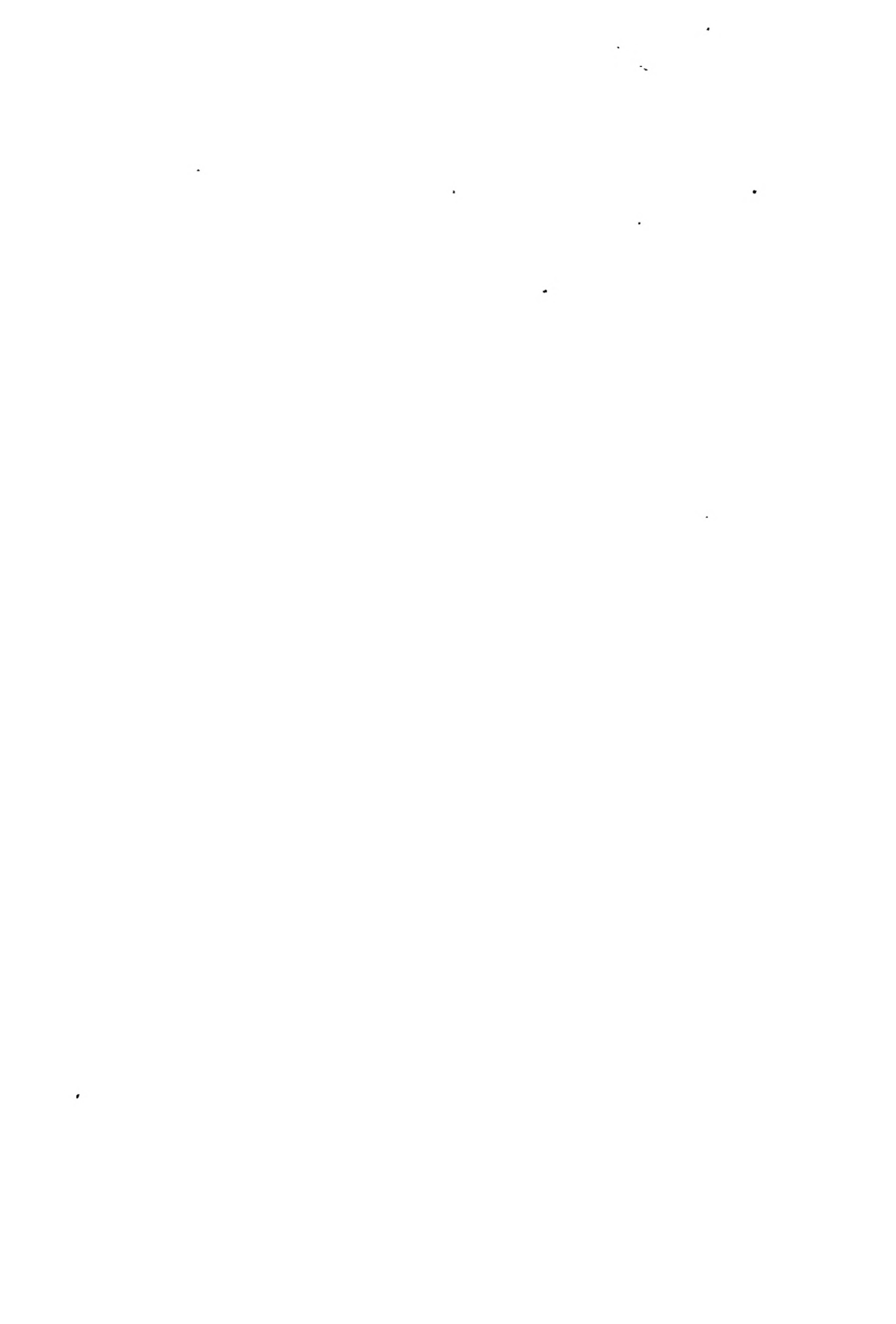
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tent of lungs of injected animals is consistent, the variation in individual rabbits of this group is evident and is not entirely accounted for by differences in the number of injections of ferric chloride. The amounts of iron in the lungs of the control animals, although definitely less than in the injected animals, show also marked individual variations. The varying intervals between the injection of tubercle bacilli and the death of the animal had no evident relation to the individual differences in iron content (*cf.* Rabbits 8, 9, and 17).

Analyses were also performed to determine the iron content of normal lung tissue in two non-tuberculous animals receiving no iron. The values obtained were 70.2 and 78.8 mg. of iron per 100 gm. of dry tissue respectively. When these two values are compared with those obtained in tuberculous animals receiving no iron (Table III) it is seen that with the exception of one case (Rabbit 8) there is distinctly more iron in tuberculous than in normal lung tissue. This is not surprising when it is recalled that in previous work (2) a greater amount of iron was likewise recovered from inflamed than from normal cutaneous areas of non-injected animals. These results with non-injected animals show that lung tissue normally contains an appreciable quantity of iron, which is evidently bound up in a form that does not give the Prussian blue reaction. It is also conceivable that in caseous areas of non-injected animals iron exists in a loosely bound form which a certain degree of postmortem change renders capable of reacting to potassium ferrocyanide.

Determinations were made on the lung tissue of two non-tuberculous animals that previously had received 12 and 20 daily intravenous injections of 5 cc. of 0.25 per cent solution of ferric chloride crystals respectively. The purpose of these additional controls was to ascertain whether iron accumulates to the same extent in normal as in tuberculous lung tissue. The values obtained were 93.6 and 119.0 mg. iron per 100 gm. of dry tissue respectively. These results definitely show that the accumulation of iron in normal lungs after repeated intravenous injections of the ferric salt is slight when compared to its accumulation in tuberculous lungs of injected animals (Table III). In this connection it is interesting to note the recent demonstration of Fowweather and Polson (10) that in normal rabbits there is a lack of an adequate reservoir of iron in the lungs. They point out that the stor-



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EXPLANATION OF PLATES

PLATE 37

FIG. 1. Lung of Rabbit 20 (see Table II). This animal received 16 intravenous injections of ferric chloride solution. The animal was killed and the lung placed in acidified potassium ferrocyanide. The Prussian blue reaction is limited to the caseous areas of tubercles. About actual size.

PLATE 38

FIG. 2. Camera lucida drawing of microscopic section of lung (Rabbit 26, Table II). This animal received 19 intravenous injections of ferric chloride solution. Iron, as shown by the Prussian blue reaction, is deposited in the caseous area. All dots at the periphery of the area of caseation are nuclei. (A hematoxylin-eosin preparation; magnified about 85 times.)





FIG. 1

Menkin and Menkin: Iron in tuberculous areas



FIG. 2

(Menkin and Menkin: Iron in tuberculous areas)

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